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Alterations in Venous Mural Structure in Autogenous Venous Grafts and Arterio-Venous Fistulae

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ALTERATIONS IN VENOUS MURAL STRUCTURE IN AUTOGENOUS VENOUS GRAFTS AND ARTERIO - VENOUS FISTULAE

by

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ABSTRACT

A technique for the replacement of diseased or traumatized arteries was developed by Alexus Carrel in 1909. With the advent of antibiotics in the early 1950's, arterial replacement or bypass by segments of autogenous veins have been used extensively especially to bypass occluded coronary arteries.

The substitution of a thin walled venous segment for a muscular, distributing artery is known to cause histological changes in the graft. The most noticeable of these long term changes are: an increase in the fibrous density of the media and adventitia, a decrease in the smooth muscle cells in the media, and the development of sub-endothelial pads of proliferative myointimal cells.

In order to distinguish the individual steps in the sequence of histological changes, 21 arteriovenous grafts were inserted into the femoral arteries of dogs. Seven femoral arteriovenous fistulae were also produced, and the venous segments proximal to them compared with the grafts to assist in the differentiation of pressure effects from those produced by surgical trauma. The grafts were performed by a modification of Carrel's end-to-end anastomosis without triangulation, while the fistulae were of the simple "H" type. Grafts were removed after 1 to 70 days and the fistulae after 1 to 56 days. Immediately prior to their removal the vessels were perfused with 1.5% Millonig's buffered gluteraaldehyde, processed routinely, and differentially

stained with Harris' hematoxylin and eosin-Y, Gomori's aldehyde fuchsin and Gomori's trichrome. Transmission electron microscopy was used to aid in the differentiation of cell types.

The findings of this research indicated that the graft developed two new layers, one inside and one outside the original graft. An inner layer of myointimal subendothelial proliferation was formed by early proliferative pads and later generalized subendothelial proliferation. The early pads were always seen in conjunction with subendothelial loss and a dense layer of marginating neutrophils. Smooth muscle cells were the primary constituents of both the early and late proliferation. Elastic tissue was seen throughout the proliferative tissue, but was concentrated along the luminal edge, where it formed a continuous lamina.

The outer layer was a sheath of dense, regular, collagenous tissue which gradually replaced the fibrin webbing that surrounded the early grafts.

The long term fistulae developed changes which were similar to those seen in long term grafts, but there were differences in the sequence and degree of change. These differences included: 1) early focal endothelial damage in the grafts, and late focal damage in the fistulae; 2) the fistulae showed no margination layers beneath their proliferative pads; 3) there was generalized proliferation and less elastic tissue production in the fistulae.

While both the grafts and fistulae were subjected to increased hydrostatic pressures, their differing sequence and degree of change

indicated that other variables, such as the vibrational stress within the fistulae and the procedural trauma to the graft, must also play a role in the development of the histological alterations.

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VITA

The author, Craig Andrew Van Der Veer, was born on June 7, 1951, in Grand Rapids, Michigan. He is the son of Dr. C. G. Van Der Veer and I. J. (Musee) Van Der Veer.

He attended parochial school throughout his elementary education, and graduated from Grand Rapids Christian High School in 1969. He continued his education at Calvin College and Seminary, Grand Rapids, Michigan, and at Trinity Christian College, Palos Heights, Illinois, graduating with a Bachelor of Arts and Biology in May, 1974.

In July of 1974, he entered the Department of Anatomy, Loyola University Stritch School of Medicine, and selected Dr. C. C. C. O'Morchoe as his advisor. While pursuing his Masters of Science in Anatomy, he was also a part time faculty member at the Department of Anatomy, Loyola University Dental School. He will continue his medical studies at Chicago Medical School.

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INTRODUCTION

The use of autogenous venous segments as replacements for diseased arteries began in the early 1900's when Alexus Carrel developed the first successful technique for grafting venous segments into the arterial circulation. At that time, however, technical advances had outstripped the advances in clinical medicine, and the use of these grafting procedures lapsed into obscurity (14). Interest in arterial replacement and bypass flourished in the early 1950's with the advent of antibiotics, and autogenous venous segments soon became the replacement of choice.

The bypass or replacement of a muscular, distributing artery with a thin walled venous segment causes alterations in the organization of the graft wall, the most noticeable of which are: an increase in the fibrotic density of the media and adventitia, a decrease in the number of smooth muscle cells of the media, and the development of subendothelial pads of proliferative myointimal cells. The fibrosis and loss of smooth muscle cells have been related to the interruption of the vasa vasorum and development of early edema (7, 36), whereas myointimal pads have been demonstrated following endothelial damage in pigs, rats, and dogs (54; 22). Szilgayi *et al.* (1973) and Wyatt and Taylor (1967) have expressed the view that surgical trauma was responsible for the endothelial damage, while others (8, 83) have tried to demonstrate that the endothelial damage was largely due to the increased pressures and shear forces to which the graft segment was subjected.

Heath and Edwards (1958) and Easterly et al. (1968) demonstrated smooth muscle proliferation in the media and intima of pulmonary vessels when pulmonary hypertension was experimentally induced in rabbits. Similar vascular pathology in response to chronic hypertension in canine carotid autografts was expected by McCabe et al. (1967), but his group did not observe these changes.

Investigators have studied the long term effects (six months to five years) of venous grafting with both light and electron microscopy (8, 12, 15, 16, 32, 34, 43, 46, 51, 58, 88). The early sequence, cause, and functional significance of these changes, however, has received little attention. The present investigation has been directed at a sequential characterization of the histological changes in autogenous venous grafts for up to 70 days after their insertion. To aid in the differentiation of the effects caused by the most obvious environmental modification, increased intraluminal pressure, from those caused by other variables of the grafting procedure, arteriovenous fistulae were also produced and compared to the graft samples and to control veins.

LITERATURE SURVEY

I. Introduction

Progressive intimal thickening of veins occurs concomitantly with age and is accentuated by venous hypertension and hemodynamic stress (50). Insertion of venous segments into the arterial circulation causes hemodynamic and intraluminal pressure changes and should be expected, therefore, to cause intimal thickening. While the origin of the reparative cells remains uncertain, there is general agreement that there are two or more cell types involved in the repair and proliferation of the venous grafts. The endothelial cell and the smooth muscle have been demonstrated to play major roles in the repair process (22, 33, 56, 77), while the roles of other cells, such as monocytes, fibroblasts, and multipotential adventitial cells, remains unsettled.

II. Endothelium of the autograft

The report that endothelial loss is of a focal nature has been well documented (8, 19, 46, 84, 88). The origin of the subsequent reparative endothelium is still debated by some. Two sources of the new endothelium have received support; endothelium derived from blood elements, and that derived from the advancing edge of adjacent endothelium.

A. Endothelium from blood elements

Late in the 1950's and in the early 1960's, Poole et al. (1958)

and Florey et al. (1961) reported the formation of a new intimal layer on woven synthetic prostheses implanted in dog aortas. These investigators believed that the new intima had been formed from blood elements because of the irregular appearance of the cell layers. Later studies using impervious synthetic prostheses (19), however, indicated that the new intima seen inside the woven prostheses had been formed by capillary budding through the porous fabric. In related studies, Tibbs (1963), Stump et al. (1963), and Baumgartener and Spaet (1970), suspended dacron hubs within impervious dacron prostheses in dog aortas. The hubs became completely endothelialized within three weeks, and myointimal cells were also reported. These findings may indicate that the same cell type was responsible for the production of both the myointimal cell and the endothelium, and that this cell was from the blood. Although these investigators noted that the anchoring sutures were free of cells at the time the tissue was examined, 3 weeks to 5 months, the sutures could have served as pathways between the prostheses and the hubs for migrating endothelial and smooth muscle cells.

B. Endothelium from an advancing edge.

The second postulated source of reparative endothelium is an advancing edge of endothelium. This theory seems to provide the simplest model consistent with the evidence. Fishman et al. (1975) denuded the endothelium of rat carotid arteries by drying and examined the endothelial repair by use of light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM), autoradiography and en-face silver staining. The results strongly suggested

that an advancing stage of mitotically active endothelial cells was responsible for the repair of large scale endothelial damage.

C. The latent period of endothelial repair.

There has been disagreement concerning the latent period between the damage to the endothelium and the initiation of repair processes, with reported intervals ranging from hours to days. Spaet and Lejnicks (1967) demonstrated a two day interval in injured rabbit carotid arteries, while others (21) working with rats, reported an interval of less than 6 hours.

III. The Smooth Muscle Cells.

A. Myointimal cell as a smooth muscle cell.

The thickened intima which develops in autogenous venous grafts has been widely reported (8, 18, 34, 83). It is composed of an endothelial layer covering a variable number of subendothelial cells which were called myointimal cells in the earlier literature, and which more recently have consistently been referred to as smooth muscle cells. Subendothelial pads of smooth muscle cells have frequently been described in large muscular and distributing arteries in areas of turbulent flow (48), but were not encountered in normal veins. Smooth muscle cells were also present in atherosclerotic occlusive lesions (2, 7, 66, 84), where morphologically they resembled the smooth muscle cells of the arterial autograft.

B. Origin of the subendothelial smooth muscle cell.

As with the origin of the reparative endothelium, the theories

concerning the origin of the subendothelial smooth muscle cell can be separated into two groups. One theory claims that the blood's elements are the source of a multipotential cell line, and the other believes that the additional smooth muscle cells come from the graft wall.

1. Smooth muscle cells from blood elements.

In work referred to earlier (4, 79, 81), subendothelial muscle cells were found on dacron hubs suspended in impervious prostheses in dog aortas. While this development seemed to indicate a possible link between blood elements and smooth muscle cells, the possibility of smooth muscle cell migration along the suture lines, as may also be the case with the endothelium, appears to be a more probable explanation. Smooth muscle cells have been shown to migrate in response to injury (54), while there is no hard evidence to link blood elements to smooth muscle cells.

2. Smooth muscle cells from wall elements.

In 1963, Buck investigated proximal segments of ligated arteries and suggested that the smooth muscle cells of the media multiplied and migrated into the subendothelial space through fenestrations in the internal elastic lamina. The proliferation and migration of pre-existing smooth muscle cells is supported by the work of McGeachie (1975) on the regeneration of crushed rat taenial smooth muscle and by several studies of the reaction of arterial walls to injury (45, 54, 66, 23, 78).

Recent research indicates that certain smooth muscle cells are capable of mitotic activity both in vivo and in vitro. Poole, Cromwell and Benditt (1971) observed mitotic figures in the media of a traumatized artery after the administration of cholchicine. Chamley et al.

(1974) cultured vas deferens smooth muscle and noted that the smooth muscle cells, which were seen to contract spontaneously in culture, lost their spontaneous contractions shortly before dividing, and regained spontaneous activity after monolayer formation. The terms dedifferentiation and redifferentiation are used by these authors, and also by Nam et al. (1974) to explain the loss and reacquisition of typical smooth muscle cell morphology before and after mitosis. These changes included a loss of typical elongated spindle shape, loss of complete basal lamina, and an apparent decrease in microfibular area. With the aid of immunofluorescence, Hoffman and Goger (1974) also studied smooth muscle cells and reported that smooth muscle cell mitotic indices were much higher for less differentiated myocytes than were seen for more differentiated, or more elongated myocytes.

IV. Proliferative lesions.

A. Proliferative lesions as a result of endothelial damage.

Proliferation is one of the prime causes of long term failure of vascular grafts and the factors which initiate proliferation have received substantial attention. While several organic substances (e.g. cholesterols and insulin) have been shown to produce proliferation of smooth muscle cells in arteries (78), these lesions and those induced by pulmonary hypertension (18, 26) all have a common denominator. This shared characteristic is the alteration of the intimal permeability barrier as evidenced by the focal loss of endothelium and medial edema. This is also consistent with the studies of Spaet and Lejnicks (1967) who induced proliferative lesions by manual endothelial trauma to

arteries of dogs. Poole et al. (1971), demonstrated similar results after passing a silk suture through rat aortas, and also demonstrated smooth muscle cell migration toward the injured area. Further evidence for the loss of the intimal permeability barrier as a causative agent of subendothelial proliferation was provided by Fishman et al. (1975), who denuded rat carotid arteries of their intima. All investigators of short term autogenous venous grafts have reported focal endothelial loss with concomitant myointimal thickening (8, 17, 31, 45).

B. The role of platelets and plasma proteins.

Several investigators have looked for a blood borne factor which may be responsible for the proliferation of smooth muscle-like cells following endothelial damage. In 1973, Weksler and Coupal found that the cell which demonstrated the greatest degree of chemotaxic activity for white blood cells and fibroblasts was the platelet. This prompted Ross and Glomset (1973) to investigate further the role of the platelet as a stimulus to smooth muscle cell proliferation. They found that platelet rich serum induced quiescent smooth muscle cells and fibroblasts to proliferate significantly in vitro. These findings correlate well with the findings of Fishman et al. (1975) that rat carotid arteries stripped of endothelium showed greatest proliferation of smooth muscle cells in segments which had the longest exposure to blood. Other investigations (21, 72) have provided further evidence that the platelet is the prime stimulator of smooth muscle cell proliferation.

C. The chalone theory.

Chalones are considered to be inhibitors of cell proliferation which are manufactured by every cell. Bullough (1965) and Robbins (1973) suggested that the loss of chalones may explain the sudden mitotic activity seen in proliferative lesions. Loss of these tissue specific mitotic inhibitors from the cytoplasm of the cell, because of disruption of membrane permeability, is presumed to cause DNA duplication and cell proliferation. This theory is interesting in view of the disruption of vessel wall permeability and subsequent damage to cell membranes seen in the early graft edema.

V. The extracellular matrix: elastin and collagen.

It has been well established that both collagen and elastin are produced by smooth muscle cells (24, 28, 66). With proliferation of smooth muscle cells an increase in both elastin and collagen might be expected. However, Jesseph et al. (1965), McCabe and Cunningham (1967), and Jones et al. (1973) reported a dimunition of elastic tissue in the grafted segments and sparse elastic tissue between the new smooth muscle cells. Brody et al. (1972) found no elastic tissue in the subendothelial pads of smooth muscle cells of grafted segments. The diminution of elastic tissue in the graft may, however, be apparent rather than real because of the thickening of the adventitia and the media. Karrer (1960) and Ross (1971, 1973) suggested that the smooth muscle cells may pass through a fibroblastic stage during which they secrete a collagenous ground substance, and secrete elastic microfibrils only after more extensive differentiation. Kadar (1960) reported large

increases in the synthesis of elastic material which were recognizable ultrastructurally after eight weeks in experimental intimal proliferation. Ross (1971) also reported finding significant amounts of elastic tissue with its characteristic 110 A diameter microfilaments in tissue cultures of rat aortic smooth muscle cells, as had Greenlee and Ross (1969). Autoradiographic studies by Ross (1971) also indicated that much of the amorphous center of the elastic tissue was produced by the smooth muscle cells, and that the central core of the elastic tissue was formed by cross-linking of the microfibrils which rimmed the amorphous non-staining centers.

VI. Histological changes in autogenous venous grafts.

After the production of autogenous venous grafts, McCabe and Cunningham (1967), Brody et al. (1972), Jones et al. (1973), and Unni et al. (1974) all described the formation of localized pads of smooth muscle cells in the subendothelial space, that is, the area between the endothelium and the internal elastic lamina. All except Brody et al. agreed that these pads were composed of collagen with a little elastic tissue in addition to the smooth muscle cells. Brody et al. reported a complete lack of elastic tissue. In addition to the presence of the pads, these authors noted that the early grafts were subject to focal endothelial loss, although they did not correlate the areas of endothelial loss with the occurence of the pads. Brody et al. (1972) reported a complete lack of elastic tissue. In addition to the presence of the pads, these authors noted that the early grafts were subject to focal endothelial loss with the occurrence of the pads.

Brody et al. (1972) also reported that the endothelial damage was limited to the first week after grafting.

These same groups of authors reported that in the early stages the media of the grafts underwent thickening due to an acute inflammatory response, with neutrophils and platelets infiltrating the intima and media from the luminal surface. Later, varying degrees of medial fibrosis were consistently found and this was interrupted by Brody et al. to be the result of local ischemia caused by the interruption of the vasa vasorum. Many of the smooth muscle cells of the media were seen to acquire large amounts of rough endoplasmic reticulum (83), and often a basal lamina which was thickened (8). These cells with variable amounts of basal lamina and large compliments of rough endoplasmic reticulum and microfilaments have been called modified smooth muscle cells and may be similar to those cells seen in the development of the early proliferative pads. Because smooth muscle has been demonstrated to produce elastic and collagenous proteins, Unni et al. (1974), and Hoffman and Goger (1974), considered that the modified cell might represent an active secretory phase of the smooth muscle cell.

After the inflammatory response which lasted from 7 to 10 days, McCabe and Cunningham (1967), Jones et al. (1973), Storm et al. (1975), Poole et al. (1971), and Unni et al. (1974) reported decreases in the numbers of smooth muscle cells in the media and concomitant increase in the amount of fibrous tissue. McCabe and Cunningham also reported that the adventitia of the early grafts was wrapped in a layer of fibrin in which many polymorphonuclear leucocytes were embedded. This

fibrin webbing became progressively less cellular and more fibrous with the age of the graft.

VII. Changes due to ischemia, pressure, and trauma.

Brody, Angel, and Kosek (1972), in an effort to differentiate the effects of increased pressure from those due to the interruption of the vasa vasorum, established 4 classes of experimental grafts; arterial and venous bypass grafts with "intact" vasa vasorum, and arterial and venous bypass grafts with vasa vasorum removed. They found that intimal proliferative pads were found in the former group, while a high degree of medial fibrosis was noted in the latter group. They considered that the pressure effects played a major role in the formation of the pads, while the vasa vasorum interruption was held responsible for the medial fibrosis. The local ischemia was thought to cause increased collagen secretion by the transformed smooth muscle cells. While their results agreed with the mainstream of opinion concerning the cause of the medial fibrosis, they did not present any method of assessing the function of the postoperative vasa vasorum.

These results were opposed by more recent work in which Storm et al. (1975) investigated autogenous venous grafts, comparing the effects of mechanical dilatation (200 mmHg intraluminal pressure) and adventitial stripping. Neither the dilated nor the stripped vein grafts appeared to differ from the long term control grafts. The control grafts were considered to have intact vasa vasorum although many of the vasa vasorum must have been damaged in even the most careful grafting procedure.

Heath and Edwards (1958) reported that increased intraluminal pressure produced by pulmonary hypertension was directly related to an increase in the number of intimal and medial smooth muscle cells, the production of proliferative pads, and the production of elastic and collagenous tissue. In investigating human coronary bypass materials, Unni et al. concurred, concluding that intimal proliferation was not due to surgical trauma, but rather to increased pressure.

Trauma, however, must also play a large role in the response of a vessel to injury. Spaet and Lejnieks (1967) used autoradiography to demonstrate mitotic figures in aortic and vena caval endothelium following focal endothelial loss due to mechanical trauma. Hoff and Gottlob (1967) noted similar results in addition to medial fibrosis after mild trauma to the aorta and vena cava of rabbits. Björkerund and Bondjers (1971) demonstrated an increase in endothelial permeability to silver salts by newly formed endothelium after mechanical trauma.

VIII. Fistulae

Stehbens (1968) produced small arteriovenous fistulae in rabbits to evaluate the endothelial damage produced by hemodynamic turbulence. Tissue samples from fistulae less than 6 months old showed ultrastructurally a mild degree of intimal thickening with fibroelastic tissue. Smooth muscle cells of the intima demonstrated separation and duplication of the basal lamina, attributed by Stehbens to endothelial injury. The venous valves, proximal and distal to the fistulae, were damaged and incomplete, while the media appeared to show little alteration. These findings were supported by those of Fallon and Stehbens

(1972). The long term fistulae (up to five years) examined by these latter authors demonstrated a loss of elastic tissue of the media and an increase in the elastic tissue between the tightly packed smooth muscle cells which were seen in the subendothelial space. The venous dilatation and thickening noted in the long term fistulae was presumed to be due to structural fatigue from vibration, a supposition repeated by Fallon et al. (1973) when he studied the effects of ultrasound on grafted venous segments.

The histological and ultrastructural changes in the fistulae were found to be similar to those seen in atherosclerotic plaques by Stehbens (1968). Fallon and Stehbens (1972) reached similar conclusions after production of lesions of the endothelium opposite carotid-to-jugular fistulae in rabbits. These lesions, termed jet lesions, were thought to be the result of hemodynamic stress.

While these findings indicate similarities in the venous mural response to grafting and fistulae production, there is little material concerning the development of change in the proximal venous segment of the fistulae.

IX. Purpose of the present work.

The goals of this research are to add to the present body of knowledge concerning the fate of autogenous venous grafts used as arterial substitutes. More specifically, this investigation focused on:

A. The development of a sequential characterization of the histological changes in autogenous venous segments used as arterial substi-

tutes for up to 70 days. Special attention has been given to three areas.

1. The early histological changes were examined to seek a relationship between the initial pathology and the subsequent histological alterations.

2. To determine whether or not smooth muscle was the primary proliferative tissue in vascular repair of damaged veins used as arterial substitutes.

3. To compare the extent of the elastic tissue response in the grafts and fistulae to the elastic tissue seen in the control samples and to each other.

B. To differentiate the effects of increased intraluminal pressure from the other variables of the grafting procedure through the examination of proximal venous segments of arteriovenous fistulae.

MATERIALS AND METHODS

I. General

The dogs used in this research were considered to be between the ages of one and two years, ranged in weight from 17 to 34 kg., and were supplied by Loyola Medical Center Animal Research Facility. Anesthesia was induced by intravenous pentobarbital at a dosage of 30 units/kg. and supplemented as necessary; heparin was administered both before (30 units/kg.) and after surgery (20 units/kg.). Eighteen autogenous venous grafts and seven "H" type arteriovenous fistulae were performed on the femoral vessels of 21 dogs. The grafts were examined by light and electron microscopy at periods up to 70 days, and the fistulae at periods up to 56 days postoperatively. The grafts and fistulae were compared with 5 control vein samples.

All dogs were catheterized and an endotracheal tube was inserted for use with a Bird mk IV respirator as needed. Surgery was carried out on supine dogs with their legs abducted and extended. The medial surface of the rear leg was shaved and washed with Betadine antibacterial scrub and 100% alcohol. The incision was made over the femoral sheath with a Bovie electrocautery unit. The sartorious muscle was retracted with four hemostats and the femoral sheath was incised, exposing the femoral and medial saphenous vessels.

II. Grafting procedures

The grafts used were 2.5 to 4.0 cm. long segments of the medial saphenous or femoral vein, depending on the individual vascular

pattern. The medial saphenous vein was preferred as implant material because more edema occured after ligation of the common femoral vein. The femoral artery was chosen as the site of implantation because of its high flow and small effective collateral circulation.

B. Graft procedure

The venous segments to be grafted were prepared by the ligation of all small tributaries with 2-0 braided silk, and the stripping of the loose outer layer of adventitia. The recipient area of the artery was also stripped of its outer layer of adventitia.

The artery was clamped proximal and distal to the implantation site with Meuller atraumatic aneurysm clips. The vein was ligated distal and proximal to the prepared area with 2-0 braided silk and removed with an iris scissors, flushed with heparinized Travenol irrigation solution (1000 units heparin/250 ml. Travenol), reversed, and laid next to its site of implantation. The artery was also transected with an iris scissors, but removal of a segment was unnecessary because its ends retracted sufficiently (2.5 to 4.0 cm.) for the insertion of the graft. Two end-to-end anastomoses, modified from the technique of Carrel (83) were performed using 9-0 and 10-0 Ethilon black monofilament nonabsorbable suture. Two mattress stitches were applied 60^{o} apart to anchor the graft, everting the edges of the graft and the artery. These stitches also served to approximate the intimal surfaces and maintain an open lumen. A continuous running stitch was employed between the mattress sutures. The retracted stubs of the transected artery were bathed constantly in 1% xylocaine and 10%

magnesium sulfate to decrease the local vasoconstriction and to assist in matching the diameter of the artery with that of the venous graft. A Zeiss 40x operating microscope with a 10x objective was used.

When the graft was in place the distal aneurysm clip was removed to allow the pressure in the graft to slowly approach that of the arterial system. The proximal clip was removed and external pressure was applied with sterile gauze for from 5 to 10 minutes to decrease the bleeding at the suture lines. The fascia around the femoral sheath was repaired with 2-0 chromic Ethilon suture and the skin was stitched with Vetafil suture and a straight needle. During every step of the procedure great care was taken to avoid damage from drying or excessive manipulation. The average time of arterial occlusion was 53 minutes.

Of the first five grafts attempted, all but one became occluded in the early postoperative period. This low initial success rate may have been due to the use of bulldog clamps instead of the atraumatic aneurysm clips used later. Following the acquisition of the aneurysm clips and a complete review of the procedure, none of the grafts or the fistulae were found to become thrombosed.

III. Arteriovenous fistulae

A. General

Seven arteriovenous fistulae of the "H" type were performed with minimal adventitial stripping in an attempt to identify histological changes which might be attributed primarily to the increase

in hydrostatic and pulsatile pressure. Denervation and the trauma associated with venous grafting were avoided and the pressure increased in the area of the vein proximal to the fistulae. These fistulae were performed on the same type of dogs as were the grafts: two dogs received a graft on the left side and a fistula on the right. The fistulae were produced in both the common and the deep femoral vessels. The deep femoral was more accessible and less prone to edema formation.

B. Operative procedure

The dogs were prepared for surgery as before. The fascia of the sartorius muscle was separated from that of the femoral sheath, and the sheath opened and retracted with Kelly hemostats. The vessels to be joined were stripped of their loose outer layer of adventitia in the immediate areas of the fistula. Small tributaries of the vein and artery were ligated with 2-0 braided silk and transected, thus freeing the femoral vein and artery for limited movement.

The vessels were then clamped with Meuller atraumatic aneurysm clips and an incision three times the diameter of the artery was made along the medial surface of the vein and artery. Four mattress sutures of Ethiflex 6-0 or Tevdak 7-0 green braided cardiovascular suture were placed 90° apart; one at each end of the incision, and one along each edge of the incision. These sutures served not only as anchors and guides for the fistulae, but also served to evert the edges and approximate the intimal surfaces. A continuous running stitch was employed between these mattress sutures. The aneurysm clips were then removed slowly from the proximal and distal vein, and

then from the distal and proximal artery. External pressure was applied until bleeding at the suture line had ceased. One half hour after the bleeding had stopped all dogs were given sodium heparin (20 units/kg) via the ventral vein of the tongue. As before, great care was taken to avoid damage to the fistulae from drying and manipulation; the area was frequently bathed with heparinized Travenol (1000 units/250 cc), 1% xylocaine, and 10% magnesium sulfate. The femoral sheath and deep fascia were repaired with 2-0 chromic suture and the skin closed with Vetafil suture on a straight needle.

IV. Preparation of tissue samples.

A. General

Sixteen successful grafts were removed at intervals of 1, 1, 2, 4, 6, 9, 21, 21, 25, 28, 35, 35, 42, 56, 63 and 70 days. Six additional grafts were found to be thrombosed at the time of removal and were not investigated further. Seven fistulae were removed after 1, 2, 4, 7, 14, 35, and 56 days. All fistulae were found to be patent at the time of their removal.

B. Removal of the grafts.

The dogs were anesthesized, and the previous incision line was reopened. The grafted material was identified by the presence of suture and the area distal to the graft was freed and the tributaries and branches were ligated with 2-0 braided silk. The artery was ligated 2 to 3 cm. on either side of the graft with 2-0 silk and catheterized proximally with Intramedic P.E. 90 polyethylene. An

eighteen gauge needle was inserted into the artery distal to the graft. The grafted segment was then perfused with 10 to 15 ml. of heparinized Travenol (1000 units/250 ml.), followed by 100 ml. of 1.5% glutaraldehyde in Millonig's buffer.

B. Removal of the fistulae.

The dogs were anesthesized and the incision was reopened. The fistulae were identified and delineated and a segment of vein 2.0 to 4.0 cm. proximal to the fistulae was freed of small branches which were ligated with 2-0 braided silk. The artery proximal and distal to the fistulae and the distal vein were ligated. The vein proximal and distal to the fistulae were catheterized with polyethylene tubing. The fistulae were then flushed with 10 to 15 ml. of heparinized Travenol followed by 100 ml. of 1.5% glutaraldehyde in Millonig's buffer.

C. Removal of the control veins.

Five femoral control veins were obtained from dogs which had grafts on the contralateral side. The outer layer of adventitia was stripped, as it was with the grafts and fistulae. The stripped segment was ligated, cannulated at both ends, and perfusion fixed as described.

V. Light microscopy.

From the grafts, fistulae, and control veins, segments were obtained for light microscopy by dividing the samples into thirds, each of approximately 1 cm. in length. These tissues were fixed and

stored in neutral buffered formalin (4%) except for a small central segment which was processed for E.M. The tissue was dehydrated in graded alcohols (50, 70, 80, 95, 100, and 100%); cleared with toluene and infiltrated in an AO Histokinette tissue processor. These segments were then embedded in paraplast embedding media using a Tissue Tech II embedding center.

VI. Electron microscopy

Autograft material prepared for electron microscopy was obtained from the central segment of the graft. Tissue samples from the fistulae were taken from an area approximately 2 to 3 cm. proximal to the fistulae, in areas subjected to greatly increased intraluminal pressure, but to minimal operative trauma. From both the grafts and the fistulae, ring shaped segments of tissue approximately 20 mm. in length were obtained. Longitudinal strips 2 to 5 mm. in width were cut from these segments, immersed in 1.5% glutaraldehyde in Millonig's buffer at $8^{o}C$ for 2 to 5 hours and washed in Millonig's buffer. The tissue samples were postfixed in 1% osmium tetroxide for an hour. After three rinses of Ringer's solution, they were dehydrated in graded alcohols (50, 70, 80, 95, 95, 100, and 100%) and cleared in three changes of propylene oxide. Infiltration was carried out in a 1:1 mixture of propylene oxide and Dupont Epon 812 resin for 12 to 18 hours under (10 torr) vacuum. The tissue was embedded in fresh catalyzed resin in beam capsules, and after sinking, was reoriented longitudinally to the capsule and polymerized for three days at $60^{o}C$.

Due to the fibrous nature of the long term grafts longer infiltration times and less viscous media were frequently used. Fluka Chemical Company's araldyte epoxy resin was also tried in place of the Epon 812, but its sectioning and infiltration qualities were found to be inferior to those of the Epon 812.

Thick sections (1u) were cut with glass knives with a Sorval Porter Blum MT_2 ultramicrotome and stained with toluidine blue containing 1% sodium borate. Thin sections were stained with 4% uranyl acetate and 1% lead citrate. The 200 mesh copper grids carrying the stained sections were examined using a Hitachi HU-11B-2 transmission electron microscope with the images retained on Kodak 3½" x 4½" high resolution plates. The plates were processed with an Arkay EM-410-4 automatic plate processor.

VII. Observations.

The grafts and fistulae samples were investigated with an American Optical binocular microscope. The intima, media and adventitia were observed for the following characteristics:

A. Intima; thickness and continuity of endothelium, presence of marginating neutrophils, composition of the proliferative pads, and mitotic figures.

B. Media; thickness, cell types, edema, collagen, elastic tissue, and the presence of inflammatory cells.

C. Adventitia; vascularity (presence of vasa vasorum), and the proliferation and organization of an outlying thrombus.

RESULTS

I. Control samples (5 samples).

The control veins presented a normal histological appearance as described by Ham (1975). The thickness of the endothelial cells was variable and was widest in the area of the nucleus, which bulged prominently into the lumen. Transmission electron microscopy (T.E.M.) revealed a continuous cell layer with many cytoplasmic extensions into the lumen, pinocytotic vesicles, and the expected complement of organelles; mitochondria, rough endoplasmic reticulum, free ribosomes, microfilaments, dense bodies, and glycogen granules. The endothelium had a single basal lamina and the cells were joined with tight junctions. The endothelial basal lamina was seen to be closely applied to the discontinuous internal elastic lamina, with a few obliquely arranged collagen fibers constituting the subendothelial space. Smooth muscle cells were not seen in the subendothelial space.

The elastic tissue bundles were readily recognizable in light microscopic samples stained with Gomori's aldehyde fuchsin and van Geisson's counter stain. These bundles stained deep purple and formed a series of discontinuous laminae arranged in concentric circles around the internal elastic lamina. The smallest, most regularly spaced bundles were nearest the lumen, and the larger, irregularly spaced bundles were in the outer media and inner adventitia. Under T.E.M. each elastic bundle was composed of two parts; an amorphous,

nonstaining portion, and a microfibrillar component. The amorphous centers were resistent to uranyl acetate and lead citrate staining and were roughly elipsoid in cross section. The amorphous component also had many short extensions into the microfibrillar areas. The areas of microfilaments outlined the poorly staining areas and are thought to be the foundation from which the amorphous centers developed (Ross and Glomset, 1974). The elastic fibers of the outer media and adventitia were frequently seen to be wrapped for up to one half of their circumference by the cytoplasmic extensions of a fibrocyte-like cell devoid of a basal lamina. These extensions contained many electron lucent vesicles and glycogen granules, but no paired vesicles and little evidence of extensive contractile filament fields were seen. Stehbens (1974) considered these to be extensions of smooth muscle cells, but their appearance was unlike that of other smooth muscle cells seen in the same areas.

The smooth muscle cells were identified by the presence of a basal lamina, rows of grouped pinocytotic vesicles, elongated nuclei, large areas of contractile filaments, and dense attachement plaques along the cell membrane. Rough endoplasmic reticulum, free ribosomes, glycogen particles, and small vesicles were seen at either end of the elongated nucleus and mitochondria were present throughout the filamentous cytoplasm. The smooth muscle cell nuclei were elongated and typically "rumpled", with a relatively open-faced nucleus, but they rarely displayed a nucleolus. The basal lamina was continuous on all the smooth muscle cells, and its duplication was not noted.

The intracellular spaces were variable in the outer media, but became smaller and more regular towards the intima. The thickness of the smooth muscle cells ranged from 3 to 6 cell layers, separated by compact bands of mature collagen and discrete bundles of elastic tissue (Fig. 1, 2).

The adventitia was composed of interwoven, compactly arranged collagen fibers and large groups of elastic bundles. The collagen fibers of the adventitia were less densely packed than those of the media. Fibrocyte-like cells were frequently seen in relation to both the elastic bundles and the collagen fibers. The width of the adventitia was 4 to 6 times the width of the combined intima and media.

II. Grafts

A. One day grafts (two samples)

The one day samples were thin walled and distended, with purplish-red thrombus surrounding them and completely filling the space between the graft and the femoral sheath. No thrombosis was seen in the lumen.

Microscopically, approximately 50% of the luminal surface was covered with a carpet of platelets and white blood cells. The majority of these marginating cells were neutrophils, although some agranular leukocytes were also present. T.E.M. showed that these areas were devoid of a continuous endothelial lining and also demonstrated the elevation of portions of the remaining endothelium by diapedesis of granulocytes and infiltration of other blood elements

(Fig. 3, 4, 5). While the layer of marginating neutrophils also contained platelets and fibrin, and could, therefore, be called a thrombus, it will be referred to as a margination layer.

The media became edematous, expanding 2 to 3 times. Red blood cells were seen throughout the intima, media, and adventitia. The cytoplasm of the smooth muscle cells was vacuolated, with fewer areas of microfilaments. Some of these cells showed karyorrhexis, with macrophages engulfing the nuclear debris. The nuclei of the smooth muscle cells in the areas of endothelial damage were club shaped (Fig. 4) and occasionally large, vesicular nuclei were also seen. These cells were considered to be either myoblasts or fibroblasts. The nuclear alterations were not seen in areas which retained their integument. The apparently normal areas comprised the remaining 50% of the luminal area and were interspersed with the damaged areas.

The adventitia was heavily infiltrated with red blood cells and was surrounded with a clot which formed a web of fibrin, entrapping RBCs, platelets and large number of neutrophils. Fusiform cells with vesicular nuclei, presumed to be fibroblasts were seen at the adventitial edges of the fibrin webbing.

B. Two day graft (one sample)

Macroscopically, the two day sample was similar to the one day sample. It was thin walled and surrounded with thrombotic material. The graft lumen was dilated compared to the neighboring artery, and the luminal surface appeared smooth. Microscopic investigation, however, revealed large (80%) areas of endothelial damage as

evidenced by neutrophils which elevated the endothelium and extended into the media, and also by dense margination of neutrophils in areas of endothelial loss.

These areas of endothelial loss were recognized by the presence of densely packed lines of neutrophils covered by a developing pad of proliferative tissue. The pads were 5 to 20 cells in thickness and occasional mitotic figures were observed close to their luminal edges. Several cell types were noted within these pads (Fig. 6):

1. The luminal surface was covered with spindle shaped cells having oval nuclei which did not bulge into the lumen as did the control vein nuclei. These cells resembled immature endothelial cells with extensive rough endoplasmic reticulum and numerous large, oval mitochondria (Fishman et al., Spaet and Lejnicks, 1967).

2. Beneath the endothelial lining were a number of cells with scant cytoplasm, round to slightly oval nuclei, and a prominent nucleolus, strongly resembling primitive mesenchymal cells.

3. Throughout the pads were scattered neutophils and occasional plasma cells.

4. Oriented with their long axis parallel to the graft lumen, spindle shaped cells with elongated nuclei were embedded in the midst of newly deposited collagen.

5. Closely applied to the edge of the internal elastic lamina was a layer of nuclear material representing the layer of neutrophils and platelets which originally formed the margination layer. Many of the neutrophils were seen invading the media from the luminal side more deeply than was observed in the one day sample.

While the endothelial surface appeared to be divided into two areas in the one day sample (damaged and undamaged), in the two day sample much of the endothelium that remained appeared to be raised by a layer of neutrophils and platelets in the subendothelial space.

The smooth muscle cells of the media underwent further changes. Their nuclei, seen to be swollen at one day, became more rounded and some were vacuolated. Although the space between the cells increased due to generalized edema, the muscle cells themselves became less distinct, obscured by the nuclear debris strewn throughout the media (Fig. 6).

The adventitia exhibited vasa vasorum filled with RBCs and was heavily infiltrated with neutrophils and plasma cells. A narrow area between the outer media and inner adventitia contained fewer leukocytes than did other areas, indicating that these cells entered the media from both the luminal and abluminal side. Free RBCs were abundant through the adventitia, especially in the outer areas where they they were trapped in the fibrin webbing of the abluminal clot. Many spindle shaped cells were also present in the area, and trichrome staining revealed the presence of collagen.

C. Four day graft (one sample)

The four day sample was more difficult to remove than the earlier samples because the thrombus around the graft had hardened and contracted. The wall of the graft was slightly thickened grossly and slightly larger in diameter than the adjoining artery. Microscopic examination demonstrated several small thrombi attached to the

wall of the graft in areas of endothelial damage, and endothelial cells were seen with extensions over the edges of these thrombi. Neutrophils were seen aligned along the internal elastic lamina beneath the thrombi, but the formation of a layer of marginating leukocytes was not observed in this sample. As with the previous sample, the nuclear debris in the media obscured the smooth muscle cells in areas of endothelial damage and leukotaxia.

The remainder of the luminal surface was covered by a continuous endothelial layer which exhibited areas of subendothelial marginating neutrophils. These cells appeared to be streaming into the media in regions where there were cells with large, pale, vacuolized nuclei, thought to be smooth muscle cell nuclei undergoing degenerative changes (Fig. 7, 8). While the actual movement of these cells could not be observed, the changes in their distribution during sequential sampling suggested the direction of their movement.

In areas devoid of both endothelium and thrombus, a thin layer of fibrin and platelets of a constant width was present attached directly to the internal elastic lamina. A similar finding has been reported by Hausenchild and Studen (1971), and Fishman et al. This layer did not attract a margination layer, nor did it initiate a massive thrombus (Fig. 9).

The cells of the media did not appear to be typical smooth muscle cells. Their shape was more suggestive of fibroblasts, with oval nuclei, irregular cytoplasm, and large, prominent nucleoli.

D. Six to nine day grafts (two samples)

The removal of the graft samples became progressively more difficult. The soft clot seen in the earlier samples became highly vascular, and the dissection was tedious due to the bleeding of the organizing thrombus. The wall in this and each succeeding graft sample was grossly thickened. The luminal diameters of the graft and artery were very similar with a slight increase in diameter in the graft's center.

The endothelium was continuous and the intima varied in width. The subendothelial space was frequently seen to contain a layer of hyalin-like material which elevated the endothelium. In these areas, two to three cell layers covered the fibrous band. These cells were neutrophils and elongated cells which may have been either myoblasts or fibroblasts.

The greatest change in the width of this segment was seen in the intima in areas of disrupted endothelium,where large numbers of cells (10 to 20 in depth) were organizing circumferentially around the lumen. These cells had nuclei which were slightly oval, with prominent nucleoli and uniformly staining cytoplasm. Many of these nuclei had the characteristic shape of smooth muscle cells as described in the control sample.

Plaques or pads of tissue were noted protruding into the lumen. They were covered with a continous layer of endothelium, beneath which were seen several layers of spindle shaped cells oriented with their long axis around the lumen. Also present were

scattered neutrophils, plasma cells, and the mesenchymal-like cells described earlier. While these cells did not meet the criteria for smooth muscle cells (no continuous basal lamina), they did present variable amounts of microfilaments, grouped pinocytotic vesicles, and a partial, but discontinuous basal lamina. Most of the cytoplasm was, however, filled with rough endoplasmic reticulum, mitochondria, golgi apparatus, and vesicles of varying sizes and densities (Fig. 10, 11).

Between the spindle shaped cells, collagen and elastic tissue were seen with differential staining. Lying along the internal elastic lamina there was a thick collagenous lamina packed with cellular debris and marginating cells. Along the abluminal side of the internal elastic lamina was a cellular mass that was oriented in irregular cords radiating from the center of the mass, nearly perpendicular to the lumen. Tissue histiocytes with packets of debris in their cytoplasm were seen throughout this area. Cells with irregular, elongated shape appeared to be moving across this fibrous strip.

The elastic fibers of the internal elastic lamina demonstrated the extent of the subendothelial proliferation, and also demarcated the media. Elastic tissue staining demonstrated small amounts of elastic tissue in the pads and in areas of less severe subendothelial proliferation.

The adventitia appeared to be separated into two layers: the inner layer was composed of collagen, elastin, and vasa vasorum from the adventitia which was retained during the grafting procedure. The outer layer was composed of granulation tissue which was beginning to replace the abluminal clot.

E. Twenty-one to 25 day grafts (three samples).

These samples were also difficult to dissect without damaging the tissue due to its high vascularity and the adherence of the femoral sheath. The luminal diameters were well matched although there appeared to be a slight central dilation. The wall was grossly thickened with some lamination noticeable. Microscopically, the luminal surface was smoother than that seen in earlier samples. The intercellular spaces appeared to have decreased in width. The intima was thickened due to several layers of smooth muscle cells. These cells were observed with electron microscopy to have dense accumulations of microfilaments, grouped pinocytotic vesicles, and a continuous basal lamina. The nuclei had acquired the characteristic shape of smooth muscle nuclei (Fig. 12).

Between the intimal cells close to the lumen the intercellular matrix was observed under light microscopy to stain with the same intensity as did the adventitial elastic tissue when stained with aldehyde fushcin. This condensed band of elastic tissue was especially evident along the luminal edge of the proliferative pads, where it formed a nearly continuous band, at times completely enclosing smooth muscle cells (Fig. 13). TEM of the subendothelial areas showed a larger number of nonstaining areas, bounded by filaments similar to those described by Ross and Glomset (1974) and by Haust et al. (1966). These centers did not, however, have the morphology typical of mature elastic tissue.

The media was densely collagenous and contained fewer smooth smooth muscle cells than were seen in any of the control samples. The

media demonstrated the greatest diameter change seen in the grafted segments, becoming twice the thickness of the control vein media. The increased thickness was due to a decrease in cellularity and an increase in fibrous tissue.

The adventitia's outer coat continued to become more fibrous with little remaining fibrin and a larger number of fibroblasts embedded between the collagenous lamina.

F. Twenty-eight to 42 day grafts (three samples).

The dissection became easier than in the earlier graft samples with the formation of a fascial plane between the graft and the femoral sheath. As was noted in all samples after 6 days, the wall was grossly thickened. This thickening appeared to reach its maximum at some time between 21 and 28 days. The walls of these grafts were thickened with white, dense, stiff tissue.

The subendothelial space was more regular in thickness than in the previous samples, consisting of four to five smooth muscle cells with narrower intercellular spaces. The first layer of cells beneath the continuous endothelium were smaller than the normal smooth muscle cells, while those applied directly to the original internal elastic lamina were of normal size. No WBCs were seen in the intima or the adventitia. The diffuse elastic tissue near the lumen continued to condense, forming an almost continuous lamina beneath the endothelium. The subendothelial space was demarcated by the new elastic-like lamina on its luminal side and the original internal elastic lamina

on its abluminal side. The proliferative pads were also compressed, emphasizing the elastic tissue between the smooth muscle cells (Fig. 14).

The media was composed of several layers of fibroblasts and an occasional smooth muscle embedded in wide bands of collagen and discrete elastic bundles. The smooth muscle cell population was not as dense as was seen in the control vein media.

The inner layer of adventitia, marked by its elastic component, was compressed with flattened vasa vasorum and elastic elements pressed into close proximity. Outside the vasa vasorum the area of fibrin webbing was infiltrated with fibroblasts and collagenous tissue, forming a dense tube pierced occasionally by vasa vasorum (Fig. 15).

G. Forty-two to 70 day grafts (four samples).

These samples were easily located and removed, and appeared to resemble grossly the 28 to 42 day samples. Microscopic investigation showed that the endothelium was continuous and normal in appearance; no leukocytes were seen beneath it. The subendothelial space was composed of several layers of muscle cells with nuclei which were more rounded close to the lumen. The smooth muscle cells were tightly packed between laminae of elastic tissue, which appeared to occupy more than 30% of the intima's intercellular space. In the media, collagen and fibrocytes were the dominant elements. The adventitia remained as described earlier; heavily collagenous outside, and more loosely collagenous with vasa vasorum and elastic tissue inside (Fig. 16, 17, 18).

All of the mural layers inside the adventitial tube were slightly more compressed and the luminal surface was more circular in these samples than was noted in the earlier samples.

III. Fistulae

A. One to seven day fistulae (four samples).

In the early samples the fistulae and the proximal venous segments were dilated and the vein was translucent, demonstrating the valves clearly. The vibration produced by the irregular blood flow was audible at three to four feet. The fistulae were surrounded by a layer of thrombus which became more difficult to dissect with time. The proximal vein was slightly distended but did not have a surrounding thrombus. Microscopically, the endothelium was continuous and appeared undamaged, although attenuated. Edema was not seen. The smooth muscle cells also appeared to be elongated and demonstrated thin, drawn out nuclei. No increase in the elastic component was noted, and the internal elastic lamina demonstrated no subendothelial proliferation (Fig. 19). The adventitia of the two and four day graft samples was similar to that of the controls, but a heavy infiltration of RBCs was seen in the seven day sample. Few leukocytes were seen among the RBCs, indicating that the RBCs were the result of contamination when the fistula was removed.

B. Fourteen day fistula (one sample).

The 14 day sample was difficult to remove because of a circumferential, organizing thrombus which bound the fistula to the femoral

sheath. When removed, the fistula was a dilated, thin walled sac with an irregular outer coating of purple/brown thrombus. Microscopically the endothelium was continuous and appeared to be normal over 80 to 90% of the wall, but exhibited slight subendothelial thickenings in several areas. These consisted of two to four layers of small cells, having rounded, deeply basophilic nuclei.

The internal elastic lamina was readily seen as a discontinuous line of discrete elastic bundles. Valves present in this proximal segment were focally thickened, exhibiting pads of spindle shaped cells. Trichrome staining and elastic tissue staining revealed collagen and elastic tissue between these cells. Pads were present only on the luminal side of the valve (Fig. 20). An occasional neutrophil was seen in these pads, as were plasma cells and round or slightly elongated nuclei similar to the nuclei of the undifferentiated cells described in the early graft lesions. No margination layers or organizing cellular masses were noted on either side of the elastic lamina. The endothelium over these areas was composed of a tightly packed layer of cells which projected irregularly into the lumen and were discontinuous.

The media again appeared stretched, with the smooth muscle cell nuclei drawn out into narrow spears. The media exhibited little thickening, and the adventitia was free of hemorrhage.

C. Thirty-five day sample (one sample).

The fistula was fused to the femoral sheath by a mass of granulation tissue. It was not possible to remove this highly vascular mass from the fistula without damaging it, so it was left intact.

Grossly, the lumen of the proximal vein looked smooth and regular, and was slightly larger in diameter distally then proximally. Microscopically, the sample exhibited an irregular increase in mural thickness, ranging from four to eight smooth muscle cells in the media to 15 to 20 cells in the subendothelial space.

While there were pads of proliferative tissue on the luminal side of the internal elastic lamina, there were no margination layers beneath the pads, as were seen in the graft samples (Fig. 21). Proliferation was seen on the luminal side of the internal elastic lamina, with the media retaining its smooth muscle cell component. The cells with rounder nuclei were generally closer to the luminal edge, while those with elongated nuclei were seen nearer the internal elastic lamina (Fig. 22). Trichrome staining revealed the deposition of collagen between cells of the pads which had rounded nuclei with dispersed chromatin. These cells appeared to be fibroblastic. Aldehyde fuchsin staining showed more of the lightly staining elastic tissue in the pads than was present in the less thickened areas. As was noted in the graft samples, the elastic tissue was densest nearest the lumen (Fig. 21).

The adventitia appeared to have undergone little fibrosis and the dense circumferential fields of collagen seen in the graft samples were not seen in these fistulae samples.

D. Fifty-six day fistula (one sample).

The removal of this sample was difficult due to the highly vascular layer around the fistulae. The fistulae was noted after

removal to be a dilated chamber made up mostly by the venous side. The walls were thickened regularly and appeared grossly to be similar to the artery. The proximal vein was thickened irregularly.

The endothelium was continuous and appeared to be undamaged in all but one area. The mural thickness was extremely variable; the thicker areas corresponded with the areas of damaged endothelium. The endothelial cells in the damaged areas were spindle shaped, but not as elongated as the muscle cells seen in other areas. The damaged area also exhibited caps of neutrophils on either side of the immature cells (Fig. 23).

The smooth muscle cells in the thickened areas were up to 30 cells deep with characteristic nuclear shape, broad fields of microfilaments, and continuous basal laminae. Between the smooth muscle cells the intercellular spaces consisted of a mixture of collagen and amorphous material (Fig. 24, 25). With light microscopy more elastic tissue was seen nearer the lumen, although there was not as much of this tissue seen in the fistula as was seen in graft samples of a similar age. Electron microscopy demonstrated the intercellular spaces to be filled with laminar arrangements of duplicated basal lamina-like material around nonstaining, irregularly shaped areas which did not look like normal elastic tissue seen in the grafts. No margination layers or dense collagenous layers lying along the luminal side of the internal ealstic lamina were seen in the fistula (Fig. 23).

In two areas of the vessel, representing less than one fourth of its luminal surface, little difference was noted between the thickness of the media of the control samples and that of the long term

fistula. The endothelium and subendothelial space in these regions did not appear to have undergone reparative processes, and also appeared unchanged in elastic and collagen content.

DISCUSSION

It is evident that the most immediate and severe alteration in the graft's structure is that of focal endothelial loss (Fig. 3, 4, 5). These foci became covered by layers of marginating neutrophils, fibrin, and platelets, which formed a deeply basophilic layer luminal to the internal elastic lamina when stained with hematoxylin and eosin. This layer will be referred to as a margination layer. Proliferation was seen in the form of pads of spindle shaped cells luminal to the internal elastic lamina, and was always seen in conjunction with the margination layers (Fig. 6, 7, 8).

Mitotic figures were seen in the pads close to the luminal surface (Fig. 6). While no mitotic figures were seen in the intact edges of the endothelium, the cells which covered the pads resembled immature endothelial cells (22, 74) (Fig. 5). Proliferation in the pads reached a maximal amount at three weeks. After three weeks the pads flattened and blended with the adjoining areas to form a more circular luminal surface.

The areas which had retained their endothelial covering showed little change in the first two weeks of implantation. Occasionally there were neutrophils and other blood elements in the subendothelial space, but they were not seen in large numbers, as they were in the margination layers. Proliferation in these areas occurred at 21 to 28 days as a generalized, even increase in the cellularity and thickness of the subendothelial space.

The proliferative cells were identified as smooth muscle cells after 21 days, but there were many cells in the pads which may have been described as either fibroblasts or smooth muscle cells (Fig. 10, 11). Between the cells of the proliferative tissue, and especially along the luminal edges of the pads, elastic tissue was seen when specifically stained for. The elastic tissue formed a continuous lamina along the lumen in these areas, and was less dense in the intercellular spaces in the centers of the pads.

Intimal damage in the fistulae appeared to be of a smaller scale and there were fewer foci of damage than were seen in the graft samples. Damage to the intima was not seen until the seventh day, and even then, it consisted of neutrophils in the subendothelial space rather than endothelial loss.

Subendothelial proliferation of smooth muscle cells was seen in the 35 and 56 day fistulae, but dense marginating layers of neutrophils seen as consistent characteristics of the pads of the grafts were not seen in the fistulae (Fig. 21, 23). The margination layers of inflammatory cells were used as an indicator of early endothelial damage. The long term fistulae also showed large areas (one quarter of their circumference) in which their mural structure was not altered from that seen in control veins.

One of the major questions which pertains to vascular repair concerns the origin of the new smooth muscle cells seen in the subendothelial space. It has been suggested that these reparative smooth muscle cells have come from blood elements (79), from multi-

potential mesenchyme (87), and from the migration and mitosis of smooth muscle cells of the media (21, 45, 54, 68, 78).

In grafted samples, it was observed that in areas of endothelial loss proliferative pads were formed on the luminal side of the internal elastic lamina above marginating layers of neutrophils. In the pads of the two and four day graft samples, mitotic figures were observed near the luminal surface (Fig. 6). The type of cell proliferating in the pads was unknown, but the same type of plaque which demonstrated spindle shaped cells at two and four days (Fig. 6, 8), demonstrated smooth muscle cells after 21 days, with few other cell types seen (Fig. 17, 18). In examining the plaques of smooth muscle cells seen in atherosclerotic lesions, Ross and Glomset suggested that arterial medial smooth muscle cells migrated into the subendothelial pads through fenestrations in the internal elastic lamina and proliferated in the subendothelial space. Kern et al. (1972) also stated that the intimal smooth muscle had its origin from the medial smooth muscle cells. The migration of smooth muscle cells toward an area of injury has been demonstrated by Poole et al., by Murry et al., and by McGeachie, as mentioned earlier, in work done on dogs and rats. As can be seen in this research, in the two day graft (Fig. 6) there are cells at the base of the plaque whose long axis is perpendicular to the lumen, possibly migrating into the plaque. A possible indication of the direction of the movement of these cells is provided by Fig. 8, where a large gap in the layer of cells upon the internal elastic lamina may have been caused

by the migration of the cells of the proliferative plaque through the margination layer. While these observations do little to establish whether the proliferative cells were from the medial smooth muscle or mesenchyme, the presence of mitotic figures in the pads, coupled with the reports by Poole and also by McGeachie that the smooth muscle cells migrate into areas of injury and then divide, indicate the strong possibility that the smooth muscle cells of the media respond to a signal of vascular trauma.

The proliferative lesions seen in the early grafts in this research appeared to have structural similarities with those described in the studies of atherosclerotic lesions (2). The pads were composed of a luminal layer of endothelial cells which appeared immature, beneath which were seen the elongated cells mentioned earlier. These cells have alternately been described as smooth muscle cells, myointimal cells, and fibroblastic cells. Lying between the internal elastic lamina and the elongated cells, focal dense layers of marginating neutrophils and platelets were seen. Ts'ao et al. (1970) found that the exposed collagen in areas of endothelial denudation provided a stimulus and a site of attachement for thrombotic material, beginning the inflammatory response with margination of neutrophils.

The margination layers were a consistent finding in each early graft sample, and the remains of these layers appeared later at the base of each proliferative pad (Fig. 6, 7, 8, 13), suggesting a relationship between the proliferation of the subendothelial smooth muscle and the formation of a carpet of neutrophils and platelets in areas of endothelial denudation. Whether the diapedesis of these

neutrophils into the media played a significant role in the migration and proliferation of the smooth muscle cells was not clear in this research, but the studies by Rutherford and Ross (1967) and Shodell et al. (1974) indicated that the exposure of smooth muscle cells to platelets and other whole serum constituents induced smooth muscle cell proliferation. Fishman et al. (1975) and his colleagues also found that the segments of rat carotid arteries denuded of endothelium for the longest time period (i.e. had the longest exposure to plasma) also exhibited the greatest degree of smooth muscle cell proliferation. The conclusions of these investigators agree well with the observations of this research. The proliferative pads observed in the early graft samples shared a characteristic shape. All had a mounded configuration with the thickest area in the center. Following endothelial loss, the endothelium is presumed to grow in from the edges, so the center of the denuded area would be the last portion to be covered by endothelium. If the stimulus for proliferation is carried within the plasma or platelets, the central segment of the denuded area would have the longest exposure to a proliferative stimulus and therefore, the greatest thickness. This theory is in agreement with this research in that mitotic activity was observed only in areas of endothelial damage, and that no mitotic figures were seen in the pads after the completion of endothelial repair. The cells contained in the pads appeared to differentiate toward a smooth muscle cell line and the pads blended smoothly with the luminal surface of the vessel wall.

Cells within the plaques bore resemblance to both fibroblastic and smooth muscle cells (Fig. 6, 7, 8). These spindle shaped cells

had large amounts of dilated rough endoplasmic reticulum, aggregations of microfilaments, and discontinuous basal lamina. Oriented with their long axis parallel to the lumen, these cells may represent smooth muscle cells which were active in the secretion of ground substance (Fig. 6, 7, 8, 12). Ross and Glomset (1973), suggested that the smooth muscle cells which were subjected to injury may re-enter a secretory phase, or pass through such a phase (following mitosis) in their differentiation as vascular smooth muscle cells. This supposition was also echoed by Wissler (1968), Nam et al. (1974), and other investigators involved with in-vitro smooth muscle cell development, who suggested that the muscle cells "de-differentiated", or lost normal smooth muscle cell morphology, before they divided, and later, "re-differentiated", regaining their normal morphology following monolayer formation. The loss of normal morphology reported by these authors included the loss of portions of the basal lamina, their microfilaments, and their characteristic nuclear shape. Unni et al. described a similar type of cell seen in autogenous venous grafts as modified smooth muscle cells, and Kern et al. (1972) simply referred to such cells as fibroblastic cells.

In the present work, after three weeks the cells of the subendothelial space demonstrated continuous basal lamina, grouped pinocytotic vesicles, cytoplasm filled with microfilaments, and a nuclear shape characteristic of smooth muscle cells. In other words, they appeared to be typical smooth muscle cells. This is in agreement with previous research in which smooth muscle cells were seen in

subendothelial proliferative lesions following mild mechanical trauma to the vena cava and jugular veins of sheep (54, 74) and with reports of smooth muscle cells seen in human atherosclerotic occlusive disease (3, 30).

The observations from the present work were consistent with the results seen by the investigators of muscle cell development, although the evidence presented here is incomplete. The smooth muscle cells of the media were not recognizable in areas of endothelial loss. In their place were rounded cells which may have migrated into the proliferative pads (Fig. 8). Mitotic figures were seen along the luminal edge of the pad and, between 6 and 10 days, cells were seen in these pads which could be described as poorly differentiated smooth muscle cells without a continuous basal lamina (Fig. 11, 12).

In the areas which did not undergo widespread endothelial loss, the endothelium was frequently raised from the internal elastic lamina by a layer of tightly packed neutrophils. These areas also underwent intimal proliferation of smooth muscle cells, but not to the degree to which proliferation was seen when marked by the fibrotic remains of a margination layer. These findings would indicate that the degree of intimal proliferation seen in the graft samples may be a direct indicator of the degree of endothelial damage.

The fistulae of less than 14 days duration demonstrated little alteration from the morphology seen in the control veins (Fig. 20). Pads of proliferative tissue were noted in the later samples, but the

margination layers seen at the bases of the proliferative pads in the graft samples were not present in the fistulae. This may be an indication that the endothelium of the proximal vein of the fistulae received less surgical trauma than did that of the grafts. The difference may also be a reflection of the intraluminal pressure of the fistulae, which was intermediate between that of the normal canine femoral vein and artery. Along the proliferative pads of the fistulae, endothelial disruption was noted after 35 and 56 days, (Fig. 21, 22) indicating that the damage to the endothelium may be a continuous process, rather than a focal event, as is seen in the graft sequences. This is consistent with the views of Stehbens and Fallon (1972), that the mural damage and subsequent repair results from vibrational stress which the fistulae produces as a result of its irregular blood flow. The vibrational damage could be viewed as an ongoing, continuous trauma which was immediately less severe than that of the graft, but which was cumulative in nature and which presented greater resistance to successful repair. The graft, on the other hand, while initially more severe in terms of endothelial damage, was more rapidly compensated.

While Brody et al. (1972) and Jones et al. (1973) reported that the mural edema seen in early samples was generalized and relatively constant throughout. In the present work, the amount of edema and inflammation was variable. The greatest amount was seen, as expected, in areas of damaged endothelium, while less severe inflammation was seen where the endothelium was apparently intact.

In these areas, neutrophils and red blood cells were seen in the subendothelial space.

The edema was followed by fibrosis which closely paralleled the areas of inflammation. Areas which had no apparent endothelial damage did not present noticeable fibrosis, while that seen in the damaged areas was noticeable. In the proliferative pads of the grafts, collagen was seen between the spindle shaped cells after three days with trichrome staining. After 20 days, the collagen was apparent throughout the pads except for the pad's luminal edge. In areas in which the sub-endothelial space was infiltrated with blood elements, a fibrotic streak of a uniform width was seen beneath the endothelium (Fig. 9). This fibrous band was thought to be caused by fibrosis of the space formed by the separation of the endothelium from the internal elastic lamina. The weakest portion of the venous wall appeared to be the bond between the endothelium and the underlying collagen and elastic lamina. Leucocytes and other blood elements were frequently seen in the subendothelial spaces, as if the mural layers had simply peeled apart, forming a blister beneath the apparently intact endothelium.

While the inner adventitia was well outlined by elastic tissue and remained vascular, the outer adventitial layer became fibrous. This fibrous sheath resulted from the infiltration of the fibrin webbing with fibroblasts. All grafts of 35 or more days duration exhibited this feature, which may have played a major role in the graft's structural integrity. McCabe and Cunningham also

noted fibrous layers around the adventitia of long term grafts, but did not speculate as to their significance.

It is interesting to note that the vasa vasorum of the grafts appeared to retain some degree of function. They were observed in almost every sample to be packed with RBCs. It was expected that removal of the loose outer adventitial layers would destroy the vasa vasorum, but in the critical first postoperative week they still had RBCs in their lumens. While it would be very difficult to assess their exact degree of function, it was apparent that a number of them retained some functional capacity.

Although the increases in collagen in the fistulae were not as dramatic as those seen in the grafts, increase collagen was seen with trichrome stain. The greatest increase was found in the proliferative pads, and in areas where the endothelium appeared to have been raised. As with the grafted samples, some areas of the fistulae showed little fibrosis, presumably because the endothelium was undamaged. This occured irregularly along the circumference of the vein, lending support to the theory that the pathology seen in the veins proximal to the fistulae was caused by turbulence and increased pressure (74, 76). It is not known whether this difference in fibrosis was due to differences in surgical trauma or hydrostatic trauma in the graft samples, but in the proximal venous segments of the fistulae where little adventitial fibrosis was seen, the trauma must be presumed to be hydrostatic.

When specially stained, the elastic tissue in the control veins was readily recognizable as a series of discrete, darkly

staining bundles of tissue forming concentric dotted lines throughout the vessel wall. In the grafts, the innermost layer, the internal elastic lamina, was used as a marker of the intimal proliferation (Fig. 13, 14). Ross and Bornstein (1969) examined the development of elastic tissue, and described it as being composed of two elements. The first of these to appear were the bundles of 100 to 110 Å microfilaments the centers of which gave rise to the second component, an amorphous center of material which did not stain with either lead citrate or uranyl acetate. This amorphous center was also referred to by Haust et al. (1965) as the "elastic matrix", and was suggested by Greenlee et al. (1966) to be formed by the crosslinkages between the microfibrils.

Jesseph et al. (1965), McCabe and Cunningham, and Jones et al. (1967) have reported a diminution of the elastic tissue of the fibrous media. This decrease may, however, be due to an increase in the thickness of the media, causing an apparent dilution of the elastic bundles. In the present study, the internal elastic lamina was apparent throughout the graft and fistulae preparations, offering a simple assessment of the degree of intimal proliferation. The presence of the internal elastic lamina was contrary to the diminution of elastic tissue which was described in the earlier investigations (32, 33, 47).

While many investigators have described the endothelial damage and proliferation of the intima following venous autografts, little attention has been given to the development of what may prove to be

the most essential component of the autograft; elastic tissue. It must be remembered that while collagen provides tensile strength, and smooth muscle provides for autoregulatory and autonomic functions, it is the elastic tissue of the arteries which must provide resilience against the pulsatile pressures within the vessels. The formation of elastic tissue has been associated with 100 to 110 A microfilaments by Haust (1965), Oakes (1971), and others (22, 63, 67). Jones (1973) reported seeing these microfilaments in the subendothelial proliferation of venous autografts and Unni (1974) mentioned ground substance as a constituent of the proliferative pads. Neither, however, noted the formation of amorphous, nonstaining centers within the clusters of these microfilaments as was observed in this research (Fig. 18). Greenlee et al. (1966), and Ross (1972) and (1971) have described similar structures in the development of elastic tissue of fetal chick ligamentum nuchae, and also in in-vitro smooth muscle cell culture.

The elastic tissue was seen in the proliferative pads of the grafts after 10 days, but did not appear in the undamaged areas until 25 to 30 days. In the 35 day graft sample (Fig. 14) the elastic tissue could be observed as a series of dense purple laminae along the luminal edge of the vessel wall, becoming less dense farther from the lumen. This may indicate that the production of the elastic tissue is related to the increased permeability of the vessel wall following endothelial damage.

The elastic tissue in the fistulae appeared to be more diffuse than that seen in the grafts. It was spread throughout the inter-

cellular spaces and was not more extensive along the luminal edge. This may reflect the pressure differences between fistulae and grafts, or perhaps the differences in the mechanism of change between the grafts and the vein proximal to arteriovenous fistulae.

The grafts exhibited changes in all three layers: the intima, the media, and the adventitia. While recent investigators have focused their attention on the intima and media (8, 32, 40, 42, 44, 51, 80, 88), a major component of the structural alteration present in the outer adventitia was mentioned only briefly (46, 83). This layer was noted in this research to be composed of dense, regular collagenous tissue which formed a fibrous sheath around the graft segments and their points of anastomosis. This sheath was readily demarcated by the elastic tissue of the inner adventitia.

The other major change was the formation of what appeared to be a new muscular layer and a new intima which extended into the vessel lumen inside the internal elastic lamina. This new vessel was complete with smooth muscle cells, an internal elastic lamina an endothelial lining, all of which were present on the luminal side of the original venous endothelium. The appearance this gave was that of a new intima and media within the lumen of the original vein. This process has been known as "arterialization" in the past, but this term is inaccurate. It implies that the vein segment which originally was transplanted undergoes a transformation which causes its pre-existing structures to acquire the morphological characteristics of an artery. While some characteristics of an artery did

appear in the grafts with time, these characteristics were produced by the formation of new structures, rather than the transformation of a pre-existing framework. The grafted segment resembled neither an artery nor a vein, but a totally different and quite predictable structure.

The two "tubes" which were formed may have some interesting functional relationships. The outer layer of dense granulation tissue was organized circumferentially forming an indistensible tube. The original adventitia remained as a highly vascular layer of tissue inside the tube of collagen. The media was irregularly fibrosed, and, because it was a venous media it was not more than five or six muscle cells in thickness. The new vessel, produced inside the discontinuous internal elastic lamina could expand and contract with the pulse pressure due to its elastic component, while the outer, indistensible adventitia would provide an absolute barrier to over-expansion and the spongy inner adventitia could provide a cushion between the two tubes.

The proximal venous segments of fistulae were investigated in an effort to isolate increases in pressure from other variables produced by the use of the grafting procedure. While the proliferative pads of both the fistulae and the grafts appeared similar after 40 or more days, it was evident from the lack of early endothelial loss and subsequent lack of margination layers that their sequences of change differed.

The grafts and fistulae ultimately produced strikingly similar structural alterations, but appeared to do so following different pathways. The grafts appeared to respond to increased pulsatile and hydrostatic forces by early, focal endothelial loss. This was followed by endothelialization and proliferative repair luminal to the internal elastic lamina. In graft areas which did not lose their endothelium early, there was a slower subendothelial proliferation. In the fistulae, on the other hand, little endothelial damage was seen in the proximal vein until the 28 day sample (Fig. 19, 20), at which point endothelial damage and proliferation was seen (Fig. 21, 22, 23). Another difference between the grafts and the fistulae was the fistulae's lack of generalized late proliferation, a prominent feature of the grafts. Because up to one quarter of the circumference of the late fistulae remained unchanged, it is presumed that the endothelial damage and proliferation of the late fistulae resulted from the increased turbulence in the proximal vein, and was not caused by surgical trauma.

CONCLUSIONS

Seventeen autogenous venous segments were used as arterial replacements and compared with proximal venous segments of seven arteriovenous fistulae and five control veins. The results of these comparisons were as follows:

A. The venous segments used as arterial replacements underwent focal endothlial loss marked by marginating neutrophils and platelets. The layers of acute inflammatory cells were covered with a pad of proliferative tissue, which demonstrated smooth muscle cells after three weeks. Areas in which the endothelium was retained appeared undamaged and underwent a slower subendothelial proliferation. The media was irregularly fibrosed and thickened. The grafts developed two new layers; a subendothelial proliferation of smooth muscle cells and an outer adventitial collagenous sheath.

In each of the grafts the following relationships were recognized:

1. A direct relationship was seen between endothelial loss, the appearance of dense margination layers of neutrophils and the production of proliferative pads of smooth muscle cells.

2. In areas which demonstrated endothelial damage, smooth muscle cells were demonstrable in the subendothelial reparative tissue as the most prominent and consistent cellular element.

3. Elastic tissue production was seen in the subendothelial space of all graft samples. It was especially prevalent along the luminal edge of the proliferative lesions. The fistulae also showed elastic tissue production, but it was not as dense nor as localized as that of the graft samples.

B. Investigation of the adventitia and intima of the arteriovenous fistulae indicated that proximal venous segments of the fistulae were not subjected to the trauma of adventitial and early intimal damage which was associated with venous autografting. The long term fistulae developed long term histological changes which were similar to those seen in the graft samples. There were, however, differences in the sequence and degree of histological alteration between the grafts and fistulae. These differences included:

1. Early focal endothelial damage in the grafts, and late focal endothelial damage in the fistulae.

2. No margination layers were seen beneath the proliferative pads in the fistulae.

3. Less generalized proliferation in the fistulae than was seen in the grafts.

4. Less elastic tissue production was seen in the fistulae than in the grafts.

While both the grafts and the fistulae were subjected to increased hydrostatic pressures, their differing sequence of change

indicated that other variables, such as the vibrational stress within the fistulae and the procedural trauma to the graft, cannot be divorced from consideration in the models used in this research.

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STAINING APPENDIX

I. Light microscopy

Following the embedding process described earlier, 5 micron sections were cut with American Optical A-800 microtome and stained with three stains; Harris's hematoxylin and eosin-Y, Gomori's aldehyde fuchsin, and Gomori's trichrome. These stains were prepared according to the following schedules.

Harris's hematoxylin	
hematoxylin crystals	5.0 gm
alcohol 100%	50.0 ml
ammonium chloride	100.0 ml
distilled water	1000.0 ml
mercuric oxide	2.5 gm

Eosin-Y	
stock solution	
5% aqueous eosin-Y	20.0 ml
95% alcohol	80.0 ml
working solution	
stock solution	1.0 pt.
80% alcohol	3.0 pt.

xylene (30 seconds)
" "
alcohol 100% (30 seconds)
" " "
alcohol 95% (30 seconds)
" " "
alcohol 80% (30 seconds)
Harris's hematoxylin (15 min.)
tap water rinse (5 min.)
acid alcohol (5 dips)
tap water rinse
eosin-Y (90 seconds)
alcohol 95% (30 seconds)
" " "

alcohol 100% (30 seconds)
" " "
xylene
xylene
mount

Gomori's aldehyde fuchsin with van Gieson's counterstain

Gomori's aldehyde fuchsin

basic fuchsin	1.0 gm
alcohol 70%	200.0 ml
hydrochloric acid (12M)	2.0 ml
paraaldehyde	2.0 ml

van Gieson's counterstain

acid fuchsin (1% aqueous)	5.0 ml
picric acid (saturated)	195.0 ml

Schedule

xylene (30 seconds)
xylene "
alcohol 100% "
alcohol 100% "
alcohol 95% "
alcohol 95% "
alcohol 80% "
aldehyde fuchsin (30 min.)
alcohol 95% (30 seconds)
alcohol 80% "
tap water rinse
van Gieson's counterstain (2 min.)
alcohol 95% (30 seconds)
alcohol 95% "
alcohol 100% "
alcohol 100% "
xylene "
xylene
mount

Gomori's trichrome

chromotrope 2-R	0.6 gm
light green (yellowish) SF	0.3 gm
glacial acetic acid	1.0 ml
phosphotungstic acid	0.8 gm
distilled water	100.0 ml

Schedule

xylene	(30 seconds)
xylene	"
alcohol 100%	"
alcohol 100%	"
alcohol 95%	"
alcohol 95%	"
alcohol 80%	"
alcohol 70%	"

tap water rinse
Bouin's fixative/mordant (55°C) (1 hour)
distilled water wash
Gomori's trichrome (15 min.)
5% glacial acetic acid rinse

alcohol 80%	(30 seconds)
alcohol 95%	"
alcohol 95%	"
alcohol 100%	"
xylene	"
xylene	"

mount

II. Electron microscopy

A. Staining thick sections - 1% borated toluidine blue

1% borated toluidine blue

toluidine blue	1.0 gm
borax	1.0 gm
distilled water	100.0 ml

Schedule

toluidine blue covering sections slide
heat until steaming
rinse with distilled water
heat to remove the water
drip xylene over sections
mount/coverslip

B. Staining thin sections

Reynolds lead citrate

lead nitrate $PbNO_3$ *	1.33 ml
sodium citrate $Na_3(cC_6H_5O_7)\cdot 2H_2O$	1.76 gm
glacial acetic acid	30.00 ml

Uranyl acetate

Uranyl acetate *	5.0 gm
distilled demineralized water	95.0 ml

* Mallinckrodt Chemicals

Schedule

uranyl acetate (10 min.)
wash with distilled, demineralized water (30 sec.)
lead citrate (5 min.)
wash with distilled, demineralized water (30 sec.)

III. Solutions

A. Neutral Buffered Formalin

40% formalin	100.0 ml
distilled water	900.0 ml
sodium phosphate monobasic	4.0 gm
sodium phosphate dibasic	6.5 gm

B. Bouin's Fixative

saturated picric acid	750.0 ml
40% formalin	250.0 ml
glacial acetic acid	50.0 ml

C. Millonig's phosphate buffer

sol A.	sodium phosphate monobasic		2.26%
sol B.	NaOH		2.52%
sol C.	glucose		5.40%
sol D.	sol A		41.5 ml
	sol B		8.5 ml
sol E.	(working solution)	sol C.	5.0 ml
		sol D.	495.0 ml

pH = 7.3
osmolarity =

EXPLANATION OF FIGURES

Figure 1. Control vein, Hematoxylin and eosin, (63x5). This photomicrograph demonstrates the endothelial cell (en) nuclei bulging into the lumen, the thickness of the media, and the loose bundles of collagen and elastic tissue in the adventitia. Smooth muscle nuclei (sm) are also seen. The bars indicate the width of the media and the adventitia.

Figure 2. Control vein, Gomori's aldehyde fuchsin, (100x6). The elastic tissue is purple (et), the collagen pink, and the cells are yellow. Note the small discontinuous bundles of internal elastic lamina along the luminal surface, and the larger bundles in the adventitia. The width of the media (m) is indicated.

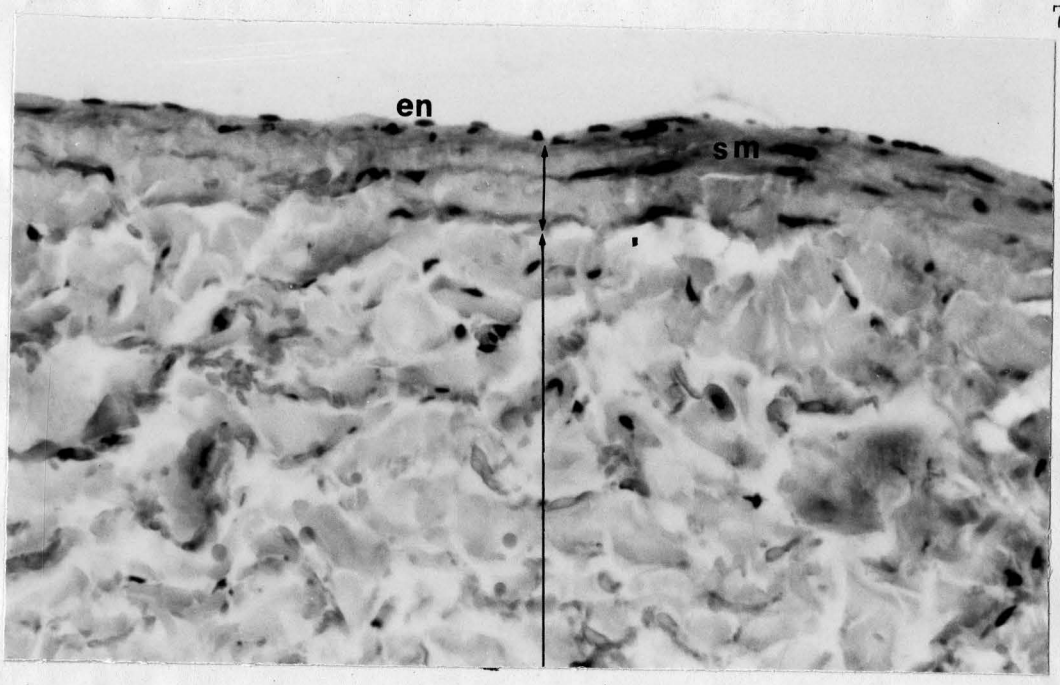


FIGURE 1

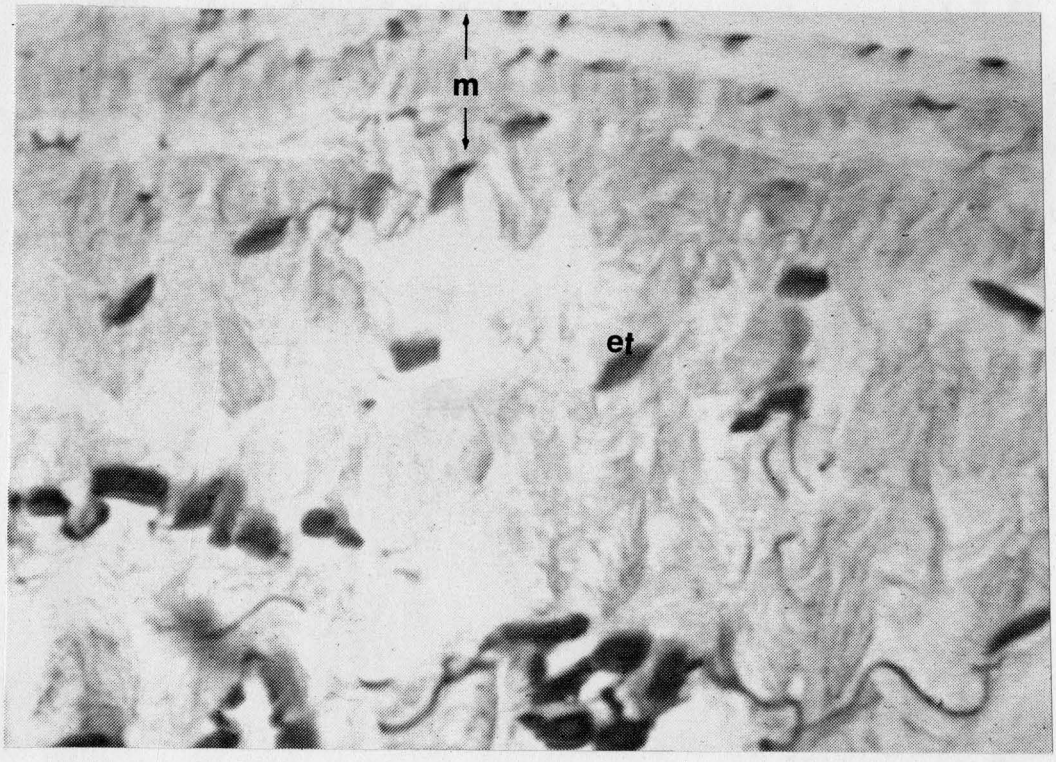


FIGURE 2

EXPLANATION OF FIGURES

Figure 3. One day graft, hematoxylin and eosin, (63 x6). The areas of endothelial damage are marked by neutrophil margination and the infiltration of the media. The adventitia (ad) is regularly infiltrated and surrounded by a clot. The clot's webbing of fibrin (fw) contains trapped WBCs.

Figure 4. One day graft, hematoxylin and eosin, (125 x5). An area of endothelial damage is marked by margination of neutrophils. The smooth muscle cell nuclei are nearly obscured by infiltrating neutrophils. Some of the smooth muscle cell nuclei (sm) have blunt, club shapped ends.

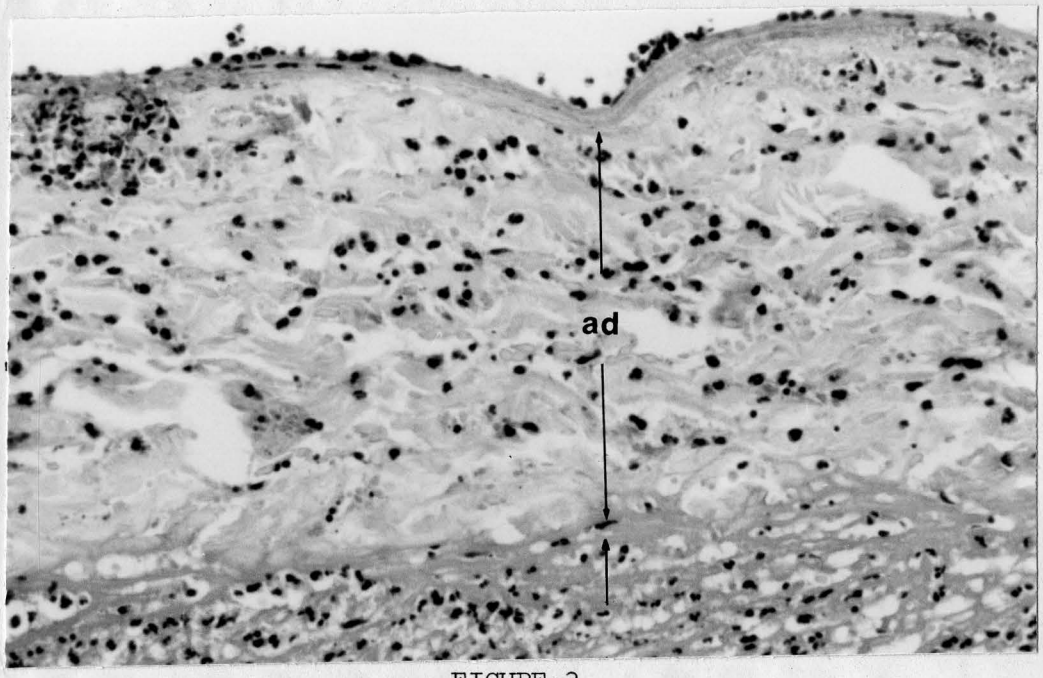


FIGURE 3

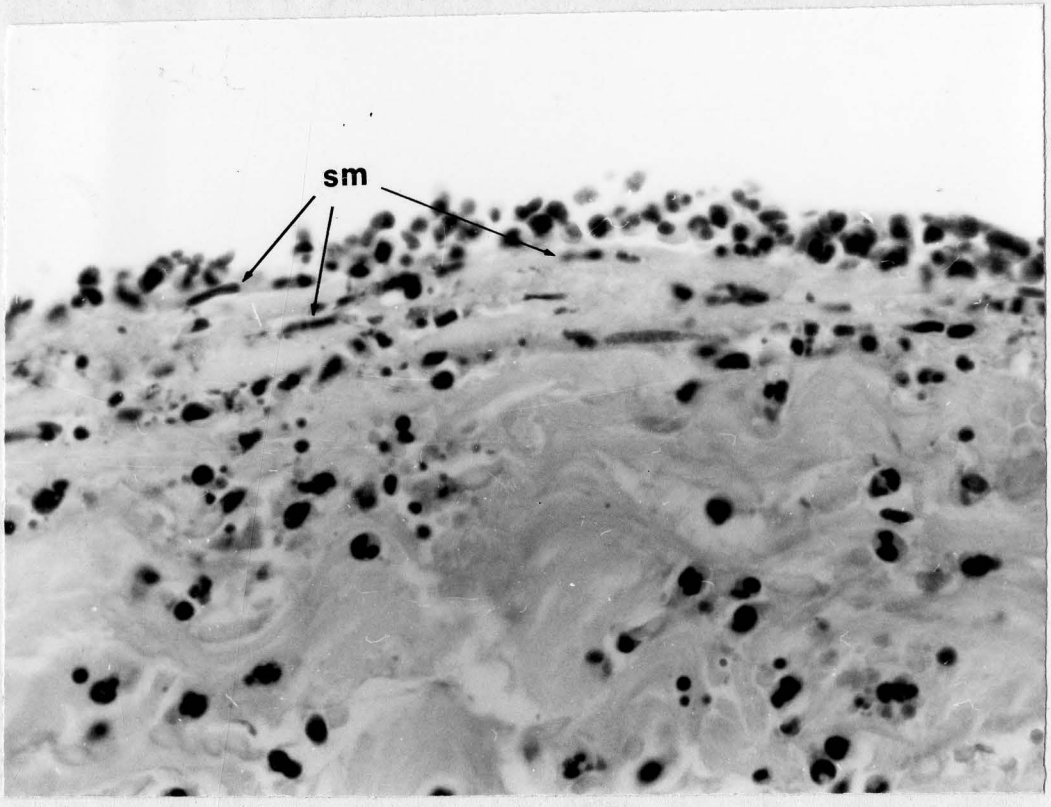


FIGURE 4

EXPLANATION OF FIGURES

Figure 5. One day graft, T.E.M., (11,500x1.5). An immature endothelial cell (en) is seen bordering an area of endothelial loss. A spear of elastic tissue(et) and strands of collagen (c) separate the neutrophils (n) and the platelets (p) in the lumen from the media. Neutrophils are also seen in the media next to a smooth muscle cell.

Figure 6. Two day graft, hematoxylin and eosin, (100 x5). A proliferative pad has formed on the luminal side of a margination layer of neutrophils. The neutrophils allign themselves along the internal elastic lamina. Movement across the lamina is seen in two areas (r). Cells of the proliferative pad include spindle shaped cells (sp), neutrophils, plasma cells, immature endothelial cells (along the lumen), and rounded to oval undifferentiated cells. A mitotic figure is seen along the lumen (mf). Cells with large, vesicular nuclei (u) are seen in the media.

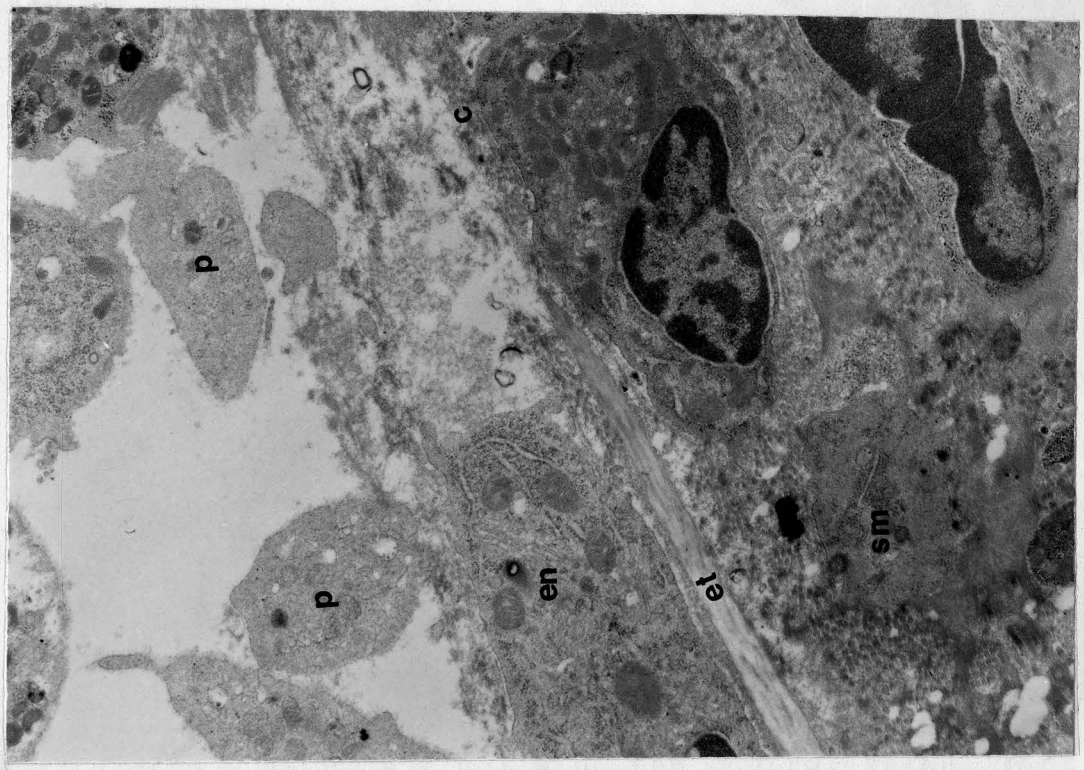


FIGURE 5

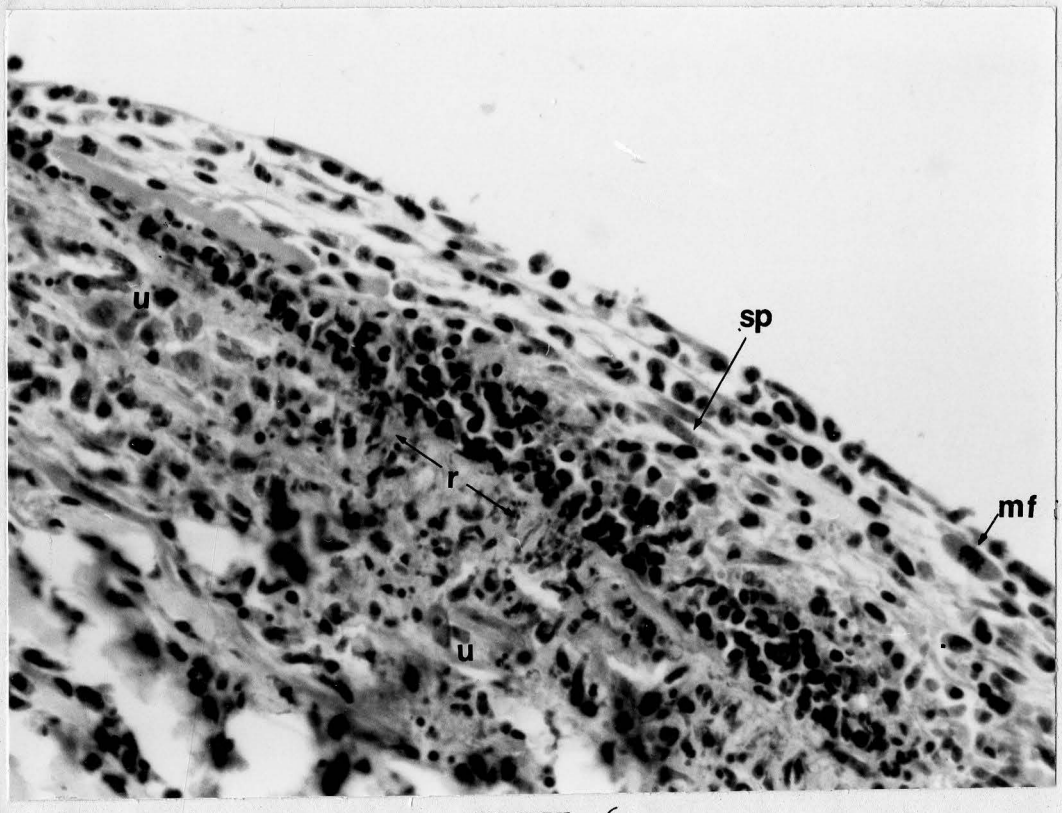


FIGURE 6

EXPLANATION OF FIGURES

Figure 7. Four day graft, hematoxylin and eosin, (63x4). A proliferative pad is seen next to an area which has undergone little structural alteration. Note that the margination layer is split in the center (arrow). Also note the highly cellular area across the internal elastic lamina from the pad. Those cells have an orientation which is perpendicular to that of the cells of the control veins. The vasa vasorum are also filled with RBCs.

Figure 8. Four day graft, hematoxylin and eosin, (100x5). This is a higher power veiw of Figure 7. The pad is filled with spindle shaped cells (sp) and covered with an incomplete endothelium. Collagen was seen in the intercellular spaces of the pad when specially stained for. The gap in the margination layer (arrow) is presumed to be the path of migration of the proliferative cells of the media of the graft. The internal elastic lamina (iel) and the nuclei of the undifferentiated cells (u) are also seen.

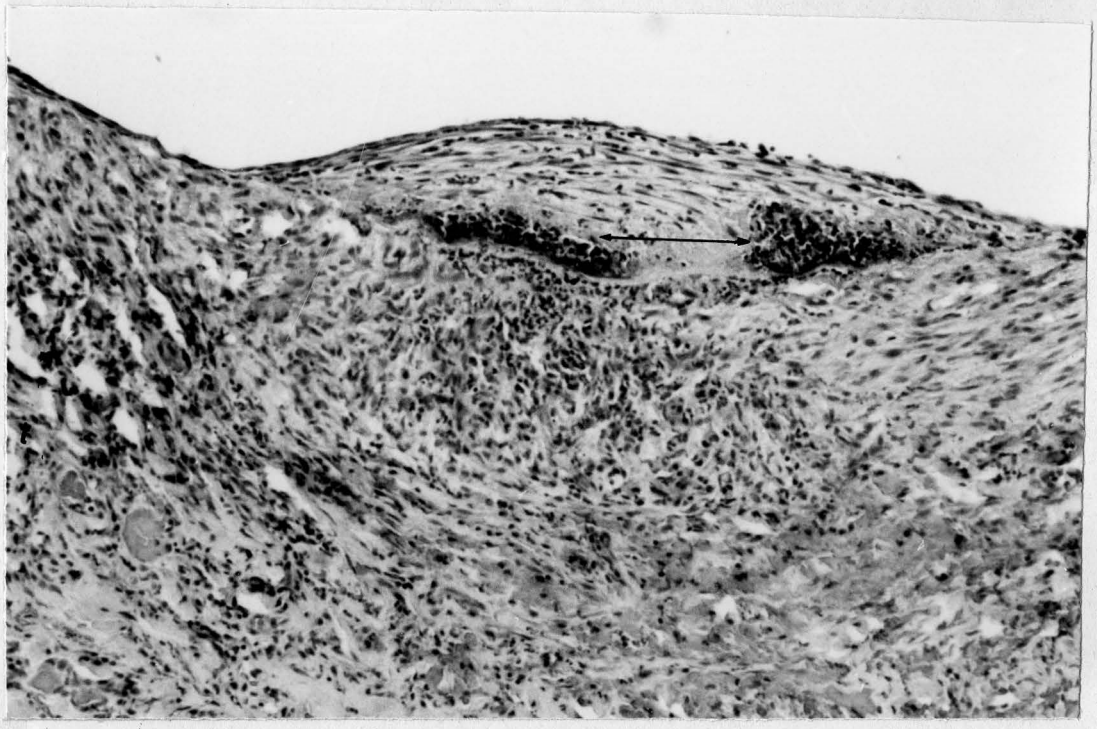


FIGURE 7

FIGURE 8

EXPLANATION OF FIGURES

Figure 9. Seven day graft, hematoxylin and eosin, (100x5). A cellular intimal layer (arrow) is suspended above the media by a band of hyaline-like material (fi). The media and the adventitia are infiltrated with neutrophils and active looking fibroblasts (fb).

Figure 10. Nine day graft, T.E.M., (10,500x4). The smooth muscle cells (sm) of the proliferative pads have large areas of microfilaments, membrane densities, rough endoplasmic reticulum (rer), and incomplete basal laminae (arrows). The intercellular spaces are filled with irregularly arranged collagen fibers(c), and irregularly staining ground substance.

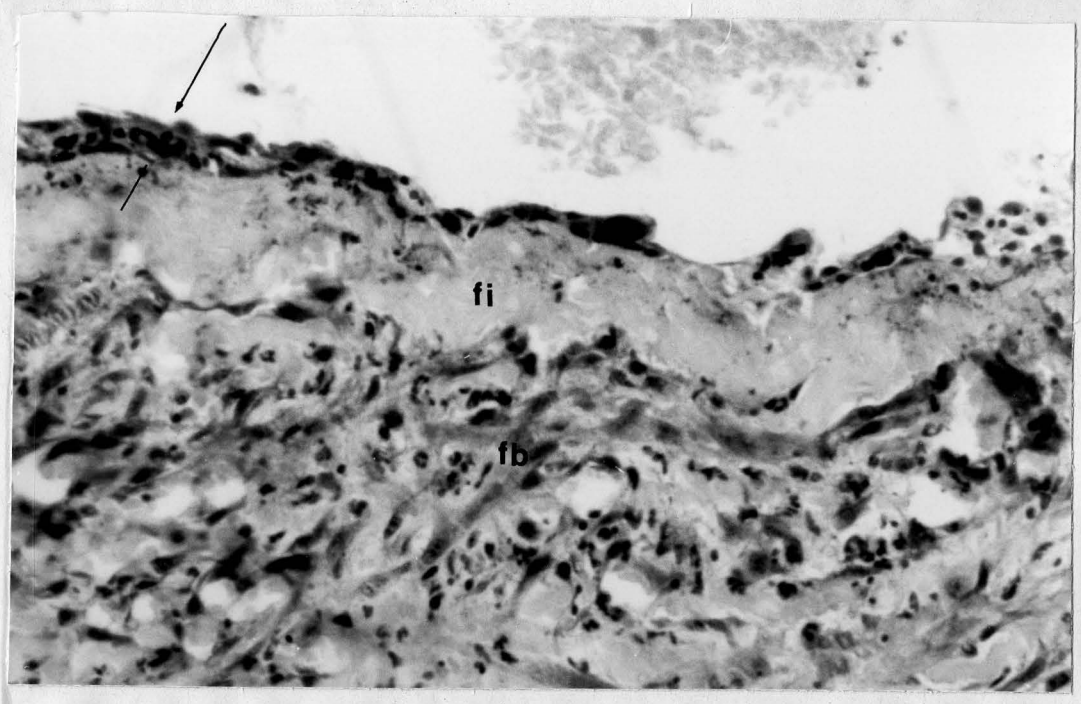


FIGURE 9

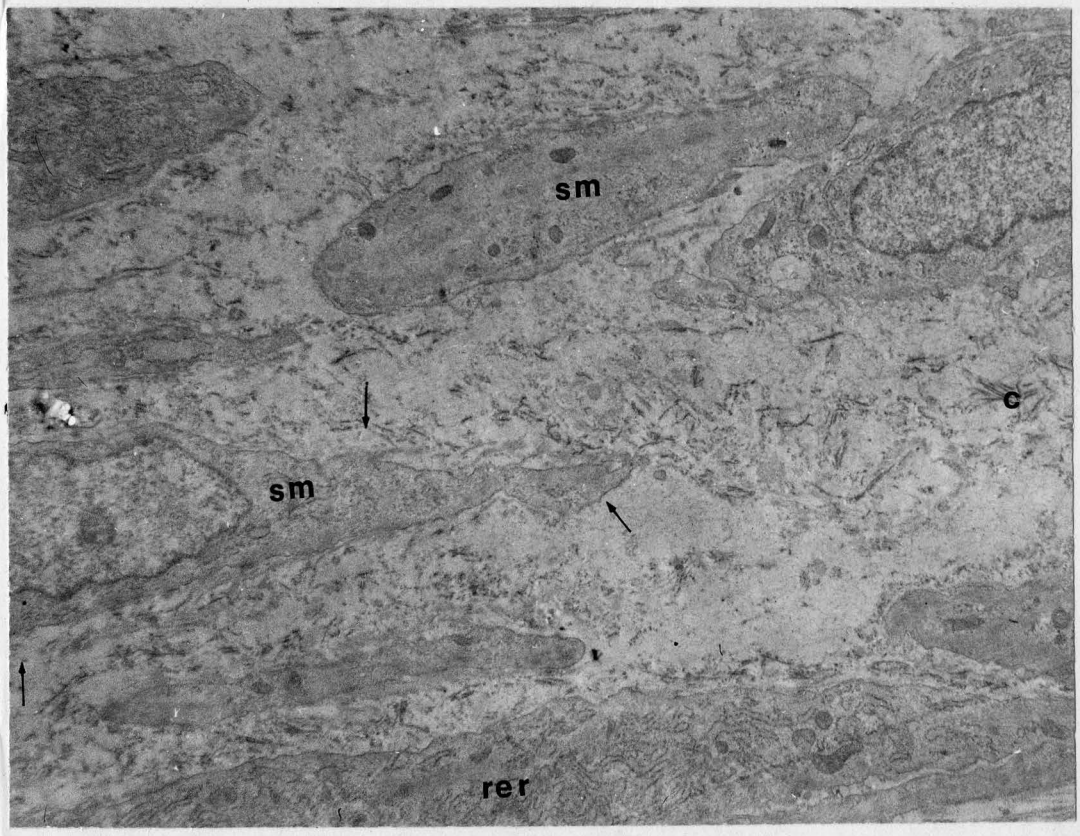


FIGURE 10

EXPLANATION OF FIGURES

Figure 11. Nine day graft, T.E.M., (23,500x2). Smooth muscle cells (sm) of the pads have incomplete basal laminae (arrows) and dilated rough endoplasmic reticulum. The deposition of collagen and ground substance appears to be more dense than is seen in Figure 10.

Figure 12. Twentyone day graft, 1u thick section, Epon, toluidine blue with 1% borate, (63x6). Cell proliferation (i) luminal to the internal elastic lamina (iel) is evident. The endothelium is continuous. A layer of dense collagenous tissue is forming abluminal to the elastic tissue of the adventitia (et). The width of the media is also indicated (m).

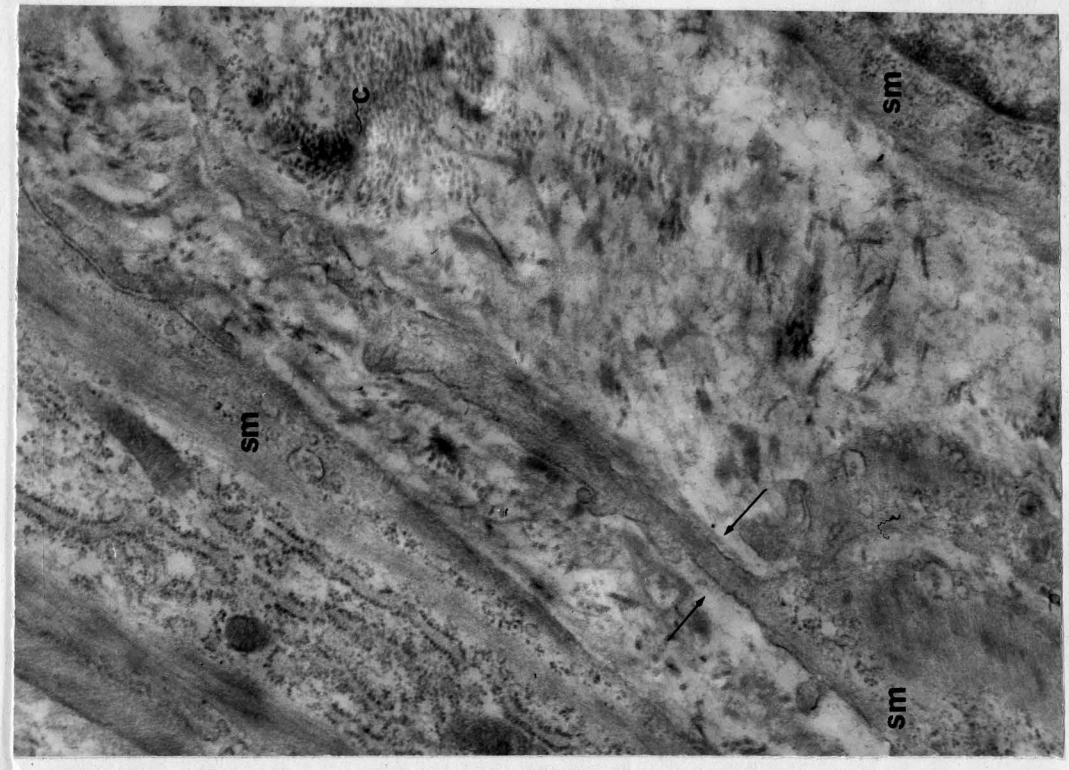


FIGURE 11

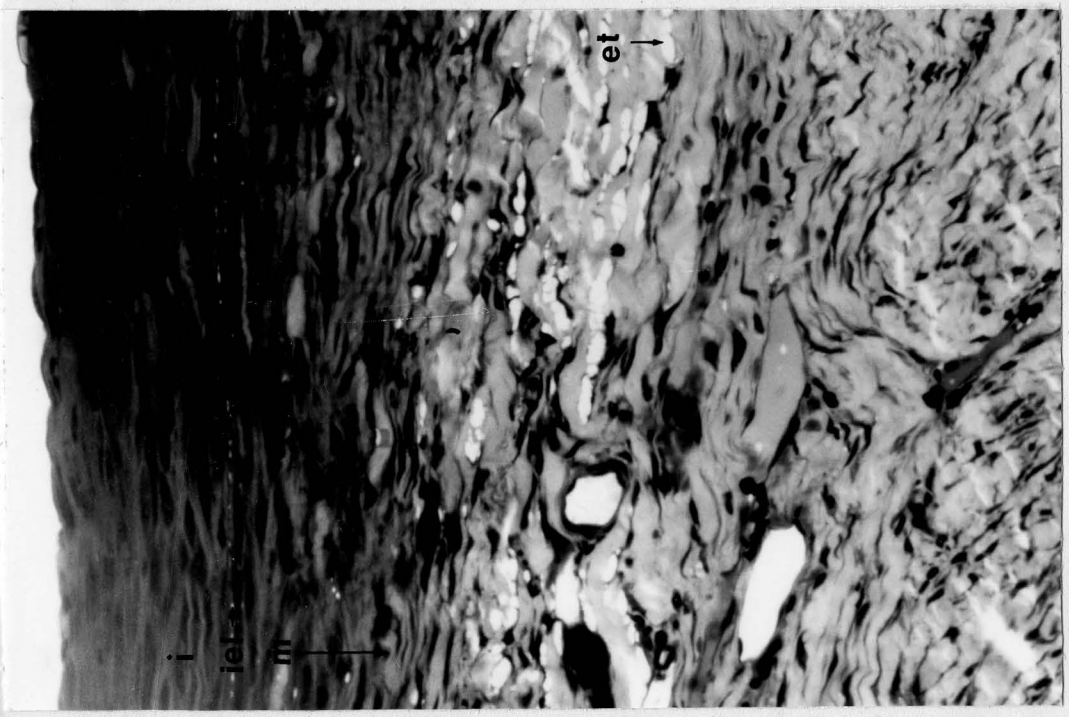


FIGURE 12

EXPLANATION OF FIGURES

Figure 13. Twentyone day graft, Gomori's aldehyde fuchsin, (100x6). A proliferative pad (i) is outlined by the purple bundles of the internal elastic lamina (iel). The cells (yellow) of the pad are outlined by diffuse (light purple) elastic tissue. The media beneath the pad is collagenous (pink) and also contains diffuse elastic tissue.

Figure 14. Thirtyfive day graft, Gomori's aldehyde fuchsin, (125x6). The production of elastic tissue (purple) is prominent along the lumen and throughout the intima (i). The smooth muscle cells (yellow) have a typical profile.

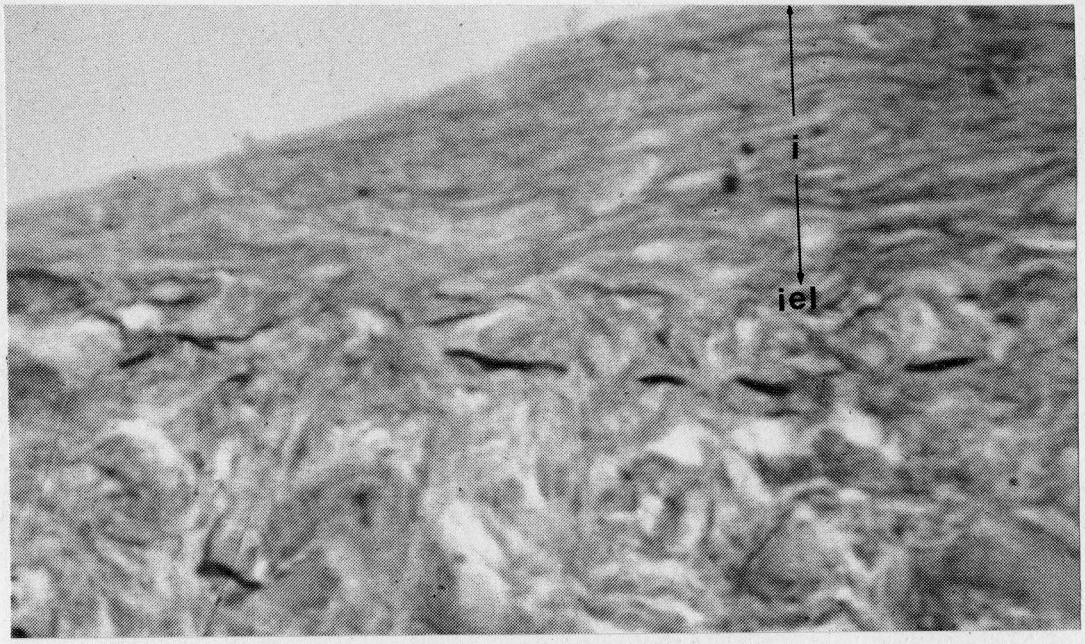


FIGURE 13

FIGURE 14

EXPLANATION OF FIGURES

Figure 15. Thirtyfive day graft, hematoxylin and eosin, (100x5). The intima is cellular with several rounded nuclei near the lumen (i). The smooth muscle cells are elongated, forming a layer 8 to 15 cells in depth. The adventitia is vascular with RBCs and a WBC in the vasa vasorum. The media (m) is thickened and fibrosed, and a dense layer of collagen (c) lies outside the adventitia (ad).

Figure 16. Fiftysix day graft, Gomori's aldehyde fuchsin, (63x6). There is a minimal amount of proliferation in the intima (i). The media (m) is fibrosed and the adventitia is well demarkated by elastic tissue bundles (purple). The outer adventitia is a densely collagenous sheath (c) devoid of elastic tissue.

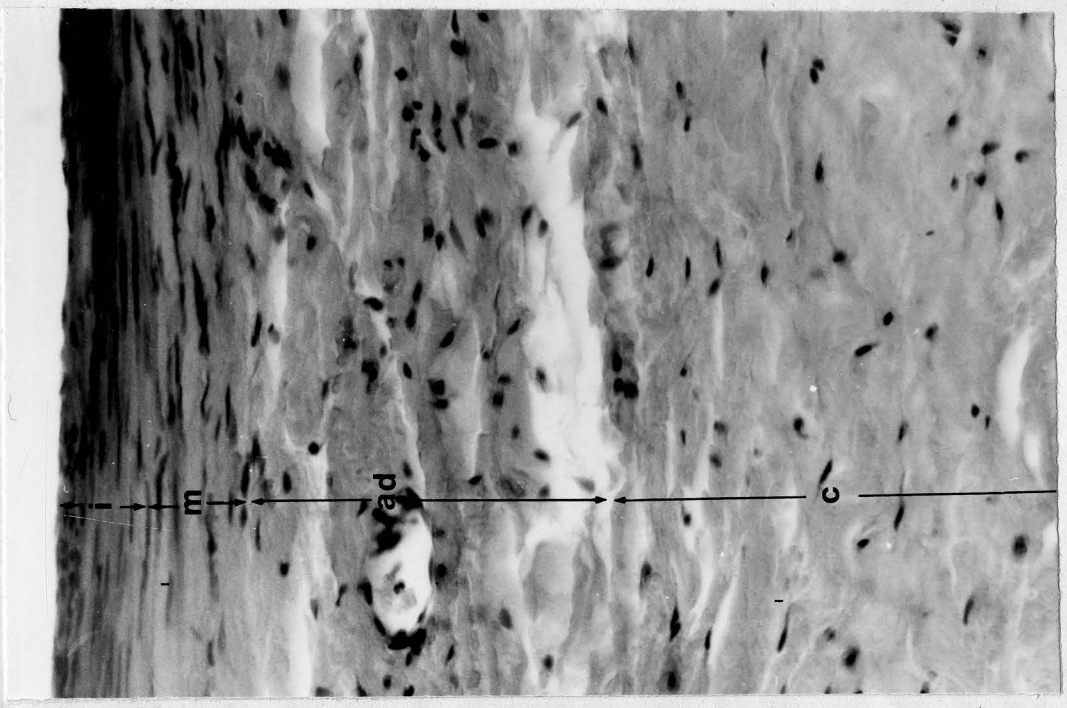


FIGURE 15

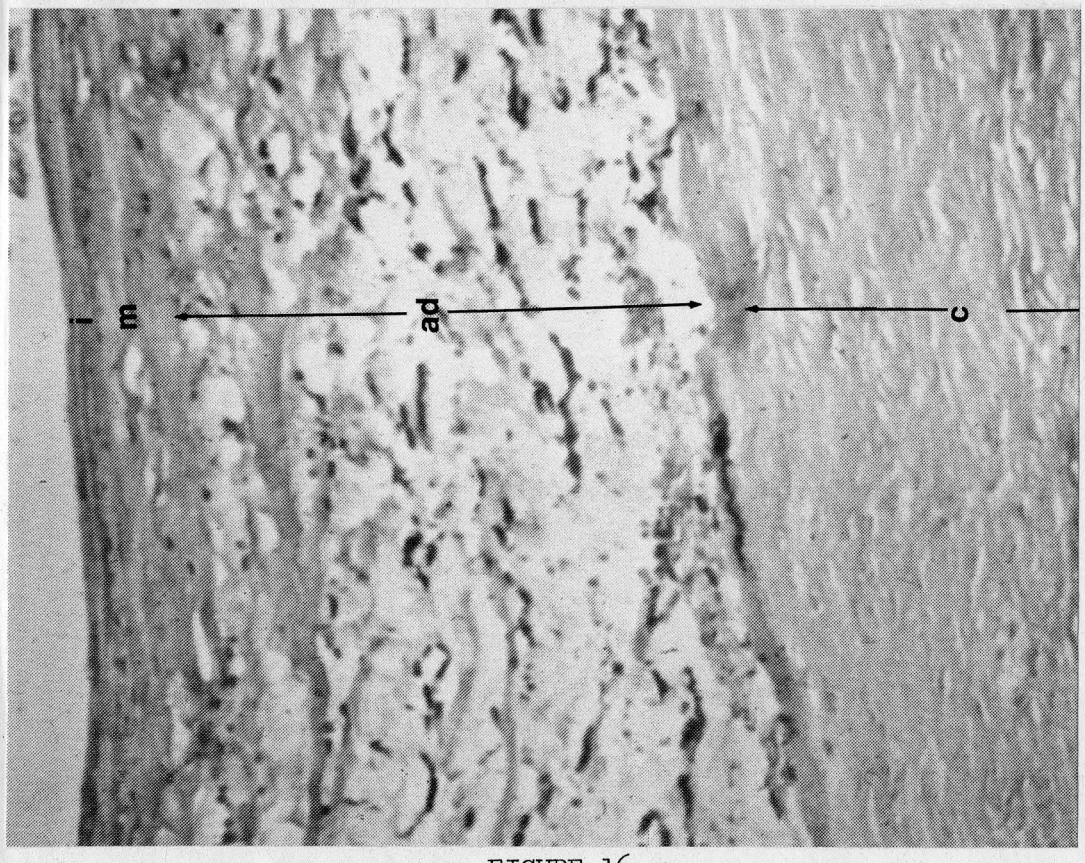


FIGURE 16

EXPLANATION OF FIGURES

Figure 17. Seventy day graft. T.E.M., (18,500x3). Elastic tissue (et) is embedded between two sub-endothelial smooth muscle cells (sm).

Figure 18. Seventy day graft, T.E.M., (27,000x1.7). Three smooth muscle cells (sm) are brodered by collagen (c) and flanked by developing elastic tissue bundles (et). The nonstaining central areas of the elastic tissue develop inside a coat of microfibrils.

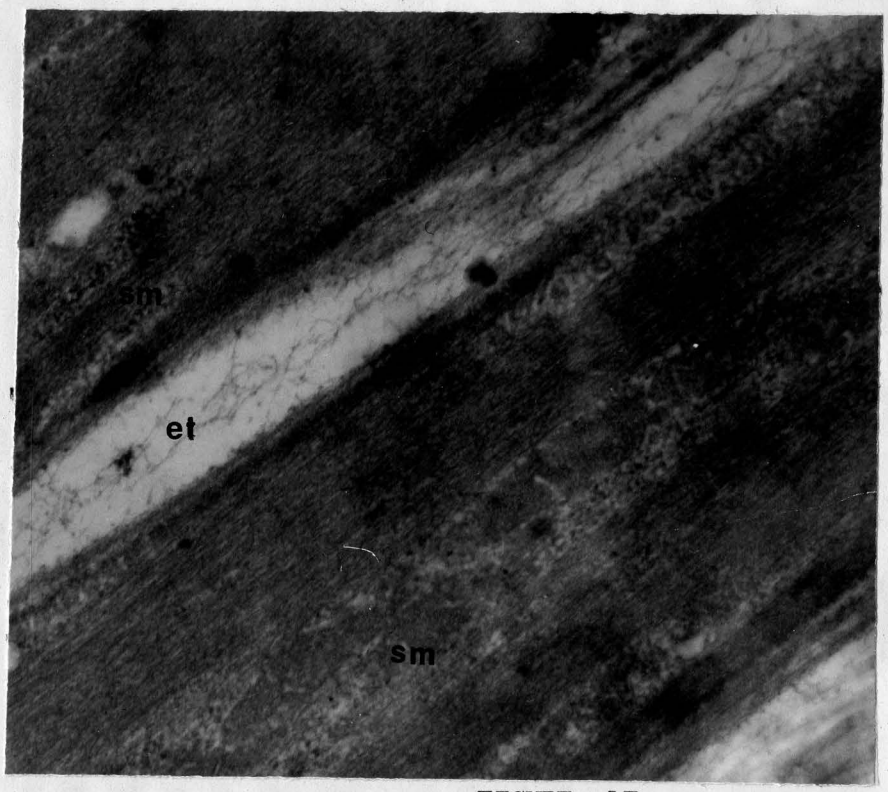


FIGURE 17

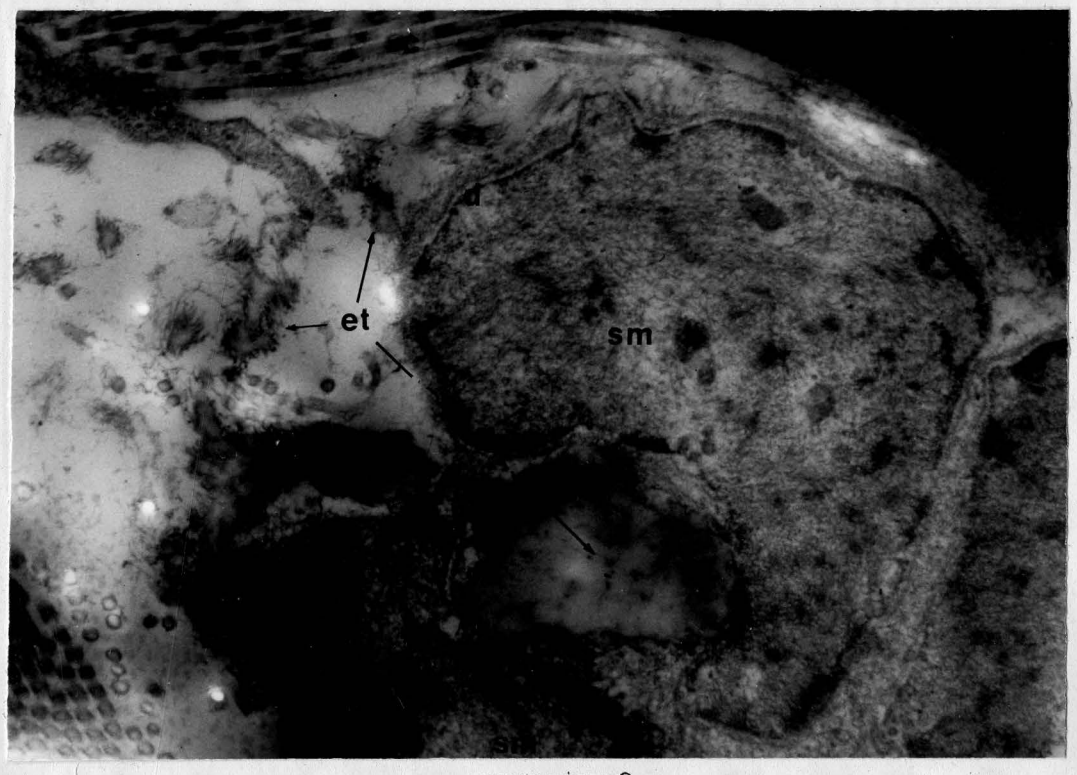


FIGURE 18

EXPLANATION OF FIGURES

Figure 19. Seven day fistula, hematoxylin and eosin, (63x5). This early fistula presents the same histology as was seen in the control veins. The intima (i) is undamaged, and the smooth muscle cells in the media (m) have retained their characteristic appearance. No inflammatory infiltration is seen, and the vasa vasorum (vv) appear to be undamaged.

Figure 20. Fourteen day fistula, hematoxylin and eosin, (63x5). The 14 day fistulae demonstrated little damage to any of the mural layers. The intima was continuous and the media was of a constant width. Proliferative areas are seen on the luminal surface of the venous valves (v).

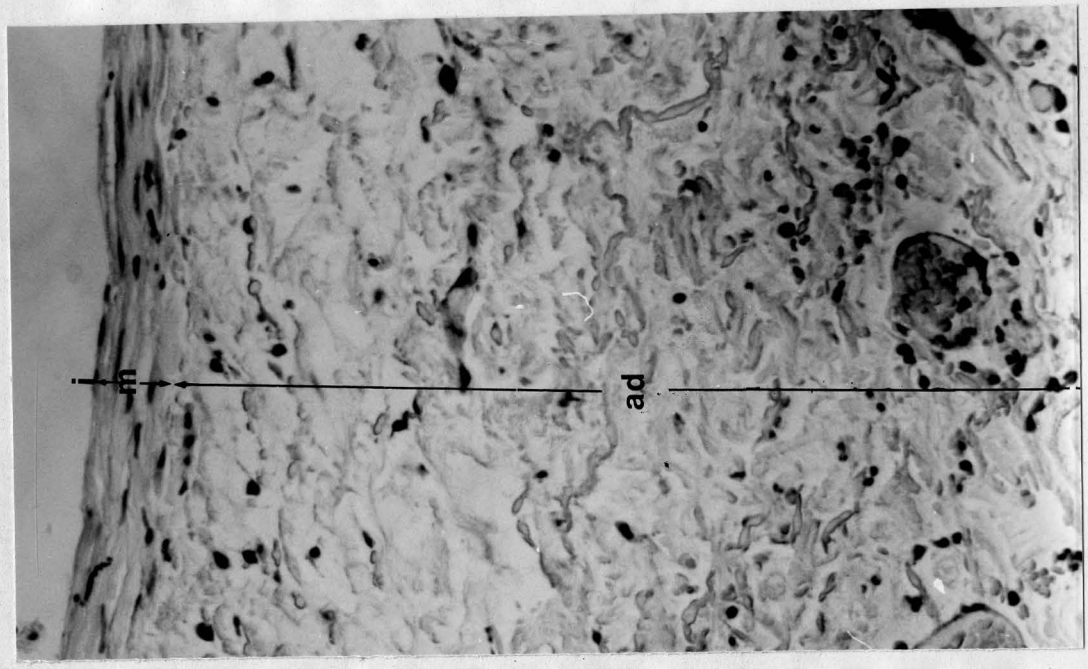


FIGURE 19

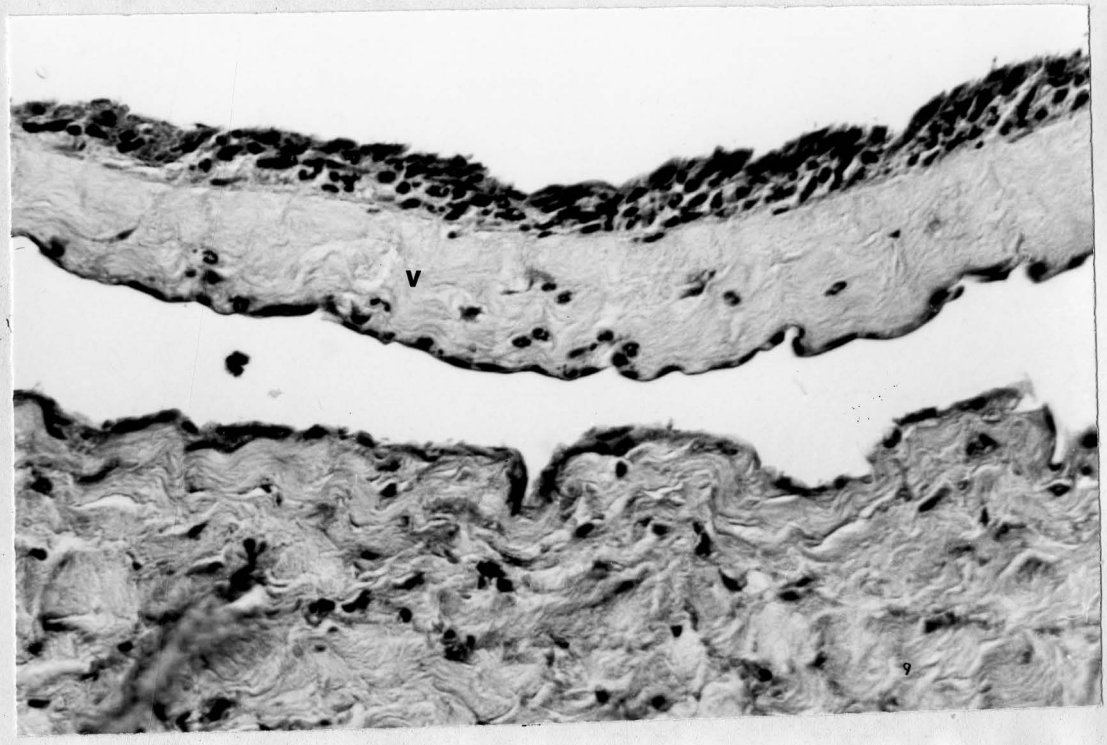


FIGURE 20

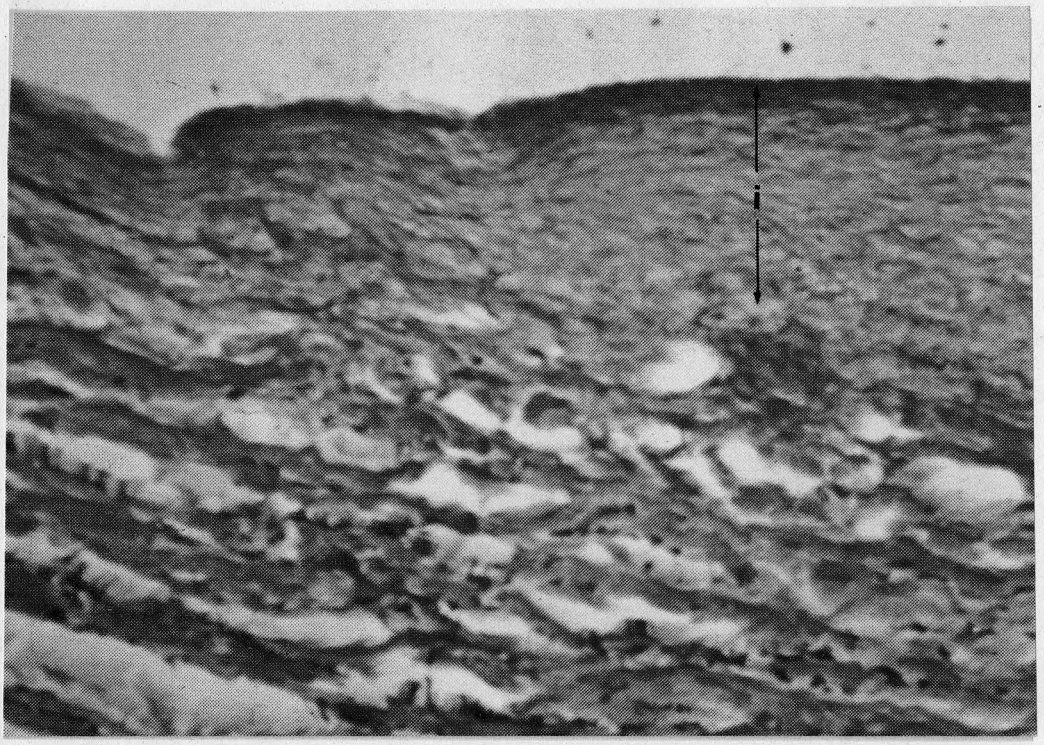


FIGURE 21

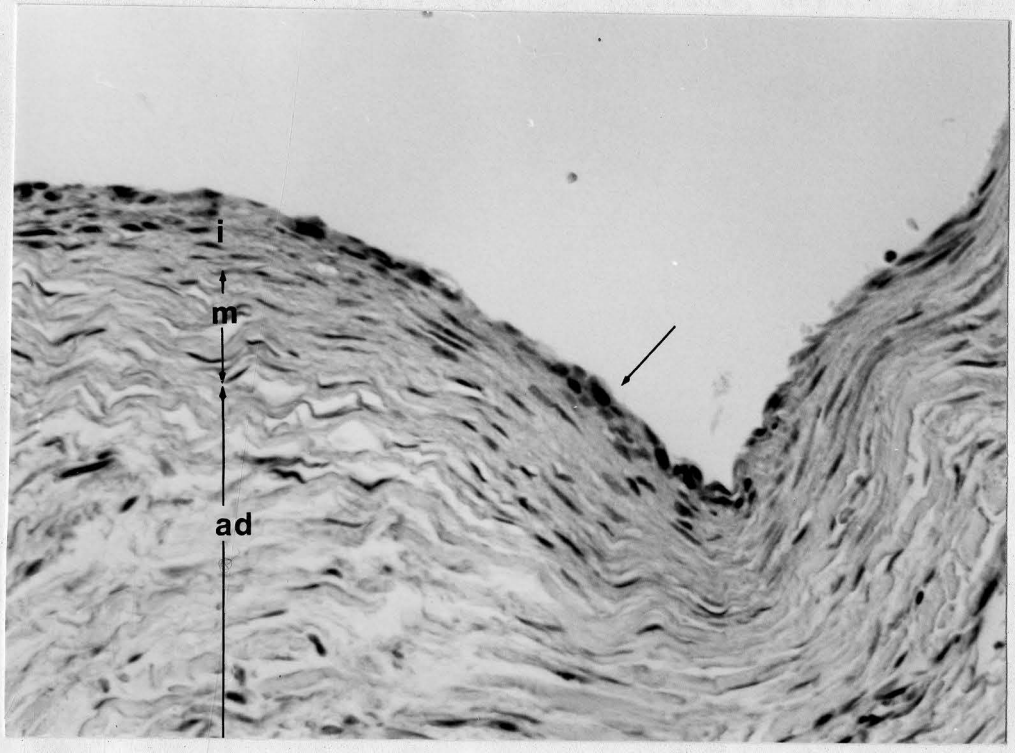


FIGURE 22

EXPLANATION OF FIGURES

Figure 21. Thirtyfive day fistula, Gomori's aldehyde fuchsin, (100x6). A proliferative pad along the lumen borders an area in which the proliferation is minimal. The junction between the intima and media is much less distinct than that seen in the graft samples because there are no margination layers.

Figure 22. Thirtyfive day fistula, hematoxylin and eosin, (63x5). Proliferation is seen along the luminal surface with rounded, apparently less differentiated cells closest to the lumen (arrow). An increase in the number of fibroblasts or fibrocytes is also seen in the inner adventitia (ad).

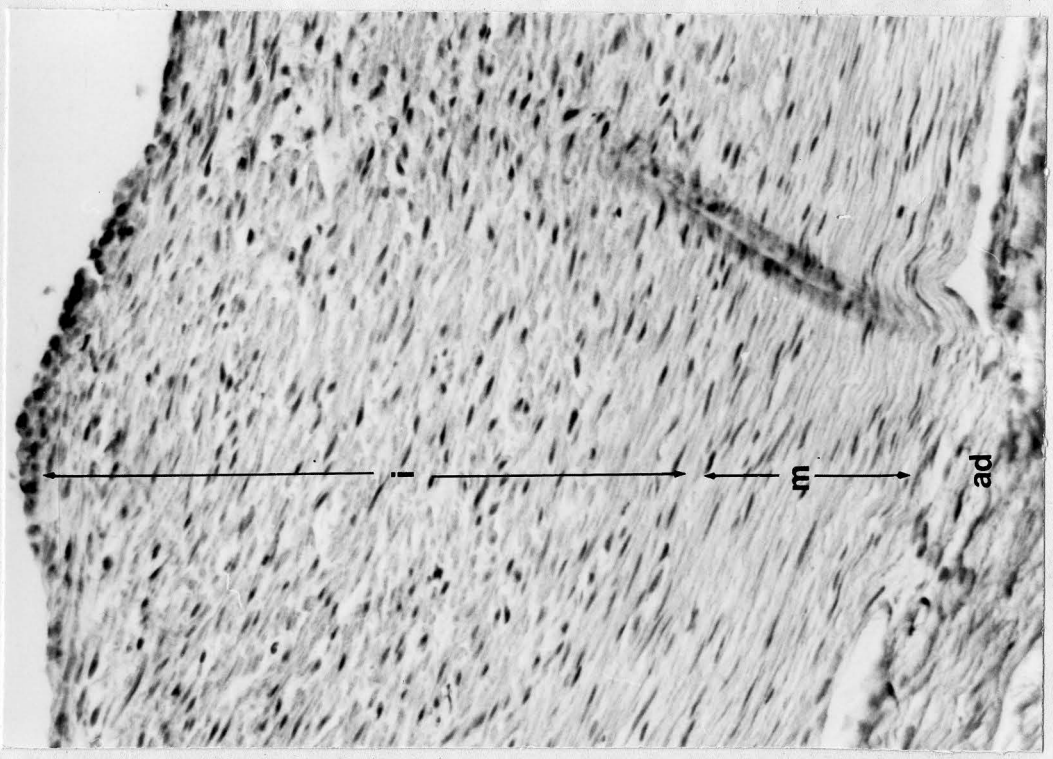


FIGURE 23

FIGURE 24

EXPLANATION OF FIGURES

Figure 23. Fiftysix day fistula, hematoxylin and eosin, (63x5). Proliferation is apparent with an increase in small, spindle shaped cells in the intima (i). The center of the proliferation is the mound of cells along the luminal surface capped with neutrophils. The media (m) is fibrosed and thickened.

Figure 24. Fiftysix day fistula, T.E.M., (28,500x3). The smooth muscle cells (sm) are flanked by laminae of elastic tissue (et). The membrane of the smooth muscle cells are less distinct than those of the graft smooth muscle cells.

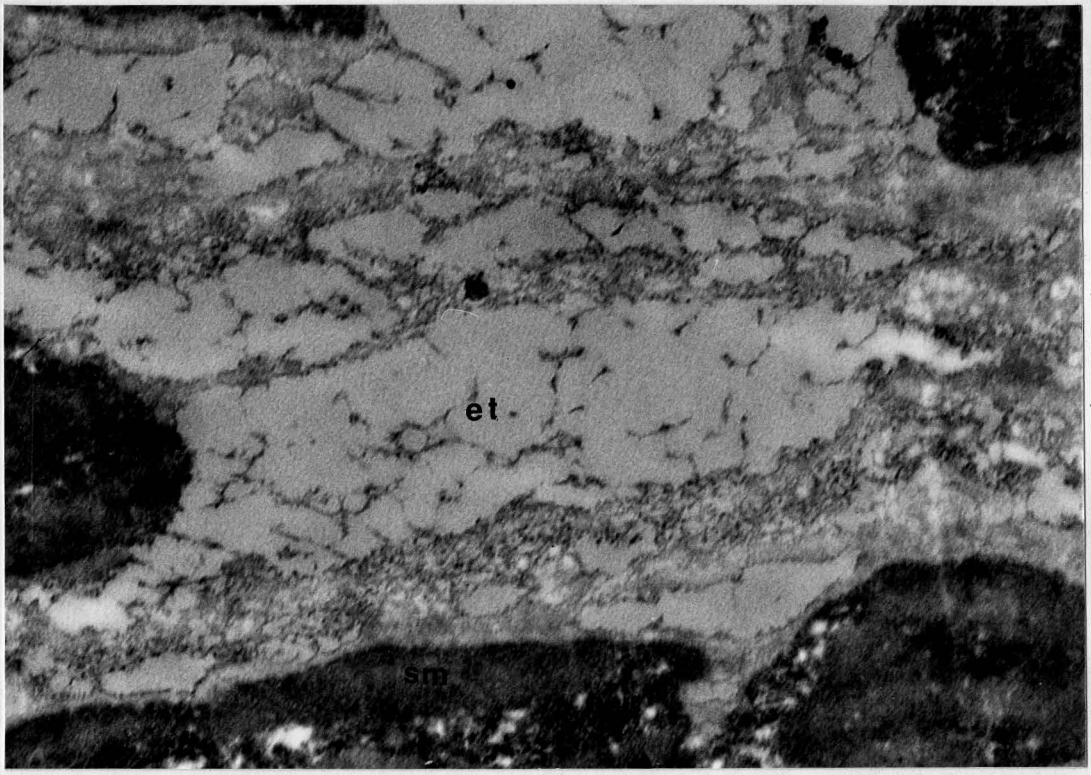


FIGURE 25

EXPLANATION OF FIGURES

Figure 25. Fiftysix day fistula, T.E.M., (28,500x4). Large aggregations of elastic tissue (et) are seen in the intercellular spaces of the proliferative pads of the fistula. The large bundles of elastic tissue are surrounded with smaller bundles of developing elastic tissue.

APPROVAL SHEET

The thesis submitted by Craig Van Der Veer has been read and approved by the following committee:

Dr. Charles C. C. O'Morchoe, Director
Professor and Chairman, Anatomy, Loyola

Dr. Leslie Emmert
Assistant Professor, Anatomy, Loyola

Dr. Raymond Warpeha
Associate Professor, Surgery and Anatomy, Loyola

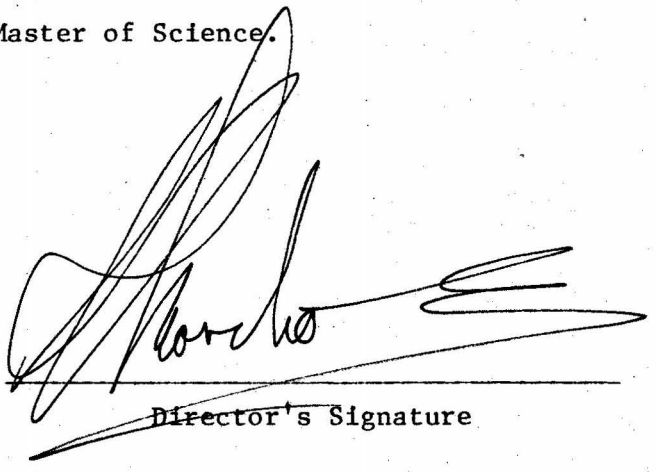
Dr. Gissur Brynjolfsson
Professor and Chairman, Pathology, Loyola

Dr. John Balis
Professor, Pathology and Anatomy, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

8/May/77
Date



Director's Signature